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The Retention of Calcium and Phosphorus by the Rat from Wholemeal Bread, with and without added Calcium, and from White Bread fortified with Calcium and Vitamin B_1

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(*Received* 30 *November* 1944)

McCance & Widdowson (1942*a*) have shown in extensive experiments that human adults retained Ca more satisfactorily from diets in which 40–50% of the calories were derived from white bread made from 69% extraction flour than from diets similar but containing bread made from 92% extraction flour. The difference was attributed to the much higher phytic acid content of the brown bread. McCance & Widdowson showed that, in order to maintain the subjects in positive Ca balance, it was necessary to add 65 mg. Ca/100 g. white flour, and 200 mg. Ca/100 g. flour of 92% extraction. The carbonate or the mono-hydrogen phosphate were equally effective as sources of Ca.

Towards the end of 1940, when these experiments had been completed but were not yet published, we had the opportunity of discussing their results with the authors. It was felt that somewhat similar experiments with young rats would be of additional interest. It was realized that the bearing of such experiments with growing rats on problems of human adult nutrition would have to be cautiously interpreted. The rat is not only specially well equipped to deal with phytate P, probably because of the possession of an intestinal phytase (Lowe & Steenbock, 1936; Patwardhan, 1937; Laskowski, 1937; Krieger, Bunkfeldt & Steenbock, 1940*a*; Giri, 1940), but its Ca and P metabolism differs from that of man in several respects, of which the refractoriness of the rat to rickets when the Ca and P intakes are normal may be quoted as an example.

EXPERIMENTAL

Plan of the experiments. The experiments were designed to compare bread made from the lowest extraction of flour available, 72%, with 100% wholemeal bread when both formed the almost exclusive diet of the experimental animals. At the time when these experiments were being planned the Ministry of Food were considering the production of white bread of 72% extraction fortified with the addition of ½ lb. of $CaCO_3$ and of 1 oz. of vitamin B_1 concentrate (equivalent to approximately 0·2 g. aneurin)/280 lb. sack (Moran & Drummond, 1940). The possibility of using purified bone meal as a source of additional Ca was also contemplated.

Two sets of experiments were therefore planned. In one the Ca content of wholemeal bread was taken as a basis and white bread was suitably supplemented with $CaCO_3$ or bone meal so that all three breads contained the same amount of Ca. In the second the comparison was with the fortified white bread of Moran & Drummond (1940). Here $CaCO_3$ or bone meal were added to wholemeal bread again to equalize the Ca content.

A third experiment was done to measure the availability of Ca from the two Ca supplements, *Creta praeparata* and bone meal. For comparison dried skim milk was also studied.

Preparation of breads. The breads were baked at the Laboratories of the Research Association of British Flour Millers (now Cereals Research Station of the Ministry of Food). Flour of 72% extraction and the wheat mixture from which it was milled were obtained from one of the members of the Association. The wheat was then ground to wholemeal and the two flours were analyzed for Ca. For baking, flour mixtures were made by addition of the requisite quantities of vitamin B_1, $CaCO_3$ or bone meal. The $CaCO_3$ was in the form of *Creta praeparata*, and the bone meal was a proprietary purified brand. After baking, the breads were sliced, crumbled and allowed to dry overnight at room temperature; they were then ground in a hammer mill.

The composition of the flour mixtures and analysis of the breads are given in Table 1.

Feeding tests. It was not possible for technical reasons to study the seven breads simultaneously. Two experiments were carried out; in the first, breads 1–5 inclusive were fed, while in the second, breads 4–7 were used. In this way breads 4 and 5 acted as a link between the two experiments. Full details of most of the experimental technique and methods of analysis will be found elsewhere (Henry & Kon, 1937). The phytate P content of the breads was determined by the method of McCance & Widdowson (1935).

Exp. 1. *The retention of* Ca *and* P *from breads* 1–5. Six litters of five male rats 23–25 days old were allocated by lot to the five breads. The intakes of breads having the same Ca content, i.e. 1, 2 and 3, and 4 and 5, were equalized, as far as possible, between litter-mates. The breads were fed mixed with 2½ times their weight of distilled water to minimize scattering. Where an animal consistently refused to eat or scattered its food badly, equalization was discontinued. Each animal received daily, separately from the bread, the following additions: 10μg. vitamin B_1, 40μg. riboflavin, 100μg. calcium pantothenate and 4 i.u. carotene. Vitamin D was not given. The animals were given distilled

Table 1. *The composition of the flour mixtures and analysis of the experimental breads*

Bread no.	Composition of flour mixture from which bread was made	Analysis of breads (% of air-dried matter) Ca	Total P	Phytic acid P	Ca/P	Moisture
1	9 lb. white flour + 1·965 g. *Creta praeparata* + 0·85 g. vitamin B_1 concentrate	0·0470	0·1115	0·0021	0·42	15·23
2	9 lb. white flour + 2·36 g. bone meal + 0·85 g. vitamin B_1 concentrate	0·0468	0·1168	0·0017	0·40	16·64
3	Wholemeal flour, no addition	0·0484	0·3465	0·1678*	0·14	14·53
4	9 lb. white flour + 7·29 g. *Creta praeparata* + 0·85 g. vitamin B_1 concentrate	0·0954	0·1174	0·0021	0·81	14·33
5	9 lb. white flour + 8·75 g. bone meal + 0·85 g. vitamin B_1 concentrate	0·0951	0·1461	0·0014	0·65	13·84
6	9 lb. wholemeal flour + 5·31 g. *Creta praeparata*	0·0983	0·3523	0·1684	0·28	13·72
7	9 lb. wholemeal flour + 6·37 g. bone meal	0·0971	0·3656	0·1683	0·27	12·95

* As the wholemeal flour contained 0·2747% phytic acid P on the dry basis it can be calculated that 29% of the phytic acid underwent hydrolysis during baking. Widdowson (1941) noticed that, in the baking of bread from flour of 92% extraction, 31% of the phytic acid was hydrolyzed.

water to drink. Excreta were discarded for the first 3 days and were subsequently collected for a period of 5 weeks. The animals were then killed, the femora and humeri removed and their ash content determined as described by Kon & Henry (1935).

Exp. 2. *The retention of* Ca *and* P *from breads* 4–7. Six litters of four male rats 23–25 days old were used; the experimental procedure was exactly the same as in Exp. 1. The intakes of the two white breads 4 and 5 and of the two wholemeal breads 6 and 7 were equalized between pairs of litter-mates.

Exp. 3. *The retention of* Ca *and* P *from dried skim milk,* Creta praeparata *and bone meal.* The diets used in this experiment were similar to diets used previously in this laboratory for comparisons between the availability of Ca from milk and from an inorganic source (Henry & Kon, 1939*a*). They were planned to supply suboptimal amounts of Ca but adequate P. The composition and analysis of the three diets used are given in Table 2. Six litters of three male rats 27–30 days old were used. The experimental procedure was essentially the same as in Exps. 1 and 2, but each rat received daily separately from the diet 10 μg. vitamin B_1, 40 μg. riboflavin, 10 μg. vitamin B_6, 300 μg. calcium pantothenate, 3 mg. choline chloride, 1 mg. inositol, 1 mg. nicotinic acid, 3 mg. *p*-aminobenzoic acid and 4 i.u. carotene. No vitamin D was given. The diet intake was equalized between litter-mates.

Table 2. *Composition and analysis of diets used in Exp.* 3

Component	Milk diet (%)	*Creta praeparata* diet (%)	Bone meal diet (%)
Egg white	11·63	15·92	16·03
Margarine fat	8·30	8·43	8·50
Maize starch	55·84	56·54	56·97
Castor sugar	7·75	7·85	7·91
Lactose	—	5·47	5·52
Ca- and P-free salt mixture	2·33	2·358	2·375
NaCl	—	0·060	0·061
KCl	—	0·051	0·052
K citrate	—	0·291	0·293
Mg citrate	—	0·103	0·104
$Na_2HPO_4.12H_2O$	1·22	2·54	1·73
Spray-dried skim milk	12·93	—	—
Creta praeparata	—	0·397	—
Bone meal	—	—	0·48
	Ca, P and moisture content of diets (%)		
Ca	0·1666	0·1677*	0·1624*
P	0·2445	0·2398	0·2372
Moisture	12·03	11·71	11·54
Ca/P	0·68	0·70	0·68

* Approximately 90% derived from *Creta praeparata* and bone meal.

RESULTS

The results of the three experiments are shown in Tables 3 and 4. The equalization of intakes proved difficult in the comparison of breads 1, 2 and 3, as the wholemeal bread was consumed much more readily than the white which the rats persistently scattered, and as a result slightly more of it was eaten. The Ca intake from this source was therefore some 10% more than from the white breads. The faecal loss was, however, also definitely higher, and, though the absolute retentions were the same, the percentage retention was significantly lower for the wholemeal bread. With the white breads the Ca was equally available from the carbonate and from the phosphate. The retention of Ca from breads 1 and 2 was lower than that observed in a previous experiment (Henry, Houston, Kon, Powell, Carter & Halton, 1941) with breads containing very much the same percentage of Ca derived from skim milk. In that experiment the Ca of added milk was available to the extent of 95% and the over-all retention from the 2% milk bread was almost 80%, that is as much as could be expected allowing for unavoidable losses of Ca in urine and faeces. The lower figure of 70% now observed is due to decidedly higher faecal losses, some 0·7 mg. daily as against 0·4 mg. for milk bread or unsupplemented white bread found by Henry *et al.* (1941). In the present experiment unsupplemented bread was not examined, and it is impossible to say whether the relatively low availability of Ca from breads 1 and 2 is due to a peculiarity of the experimental animals or to a lowering

Table 3. *Mean data* for the Ca and P metabolism of the rats in the three experiments*

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Source of Ca	Diet intake (g.)	Ca intake (g.)	Ca excretion (g.)		Ca retention		P intake (g.)	P excretion (g.)		P retention		Wt. of rat (g.)		mg. Ca intake/ g. gain	mg. P intake/ g. gain	Bone ash (%)
			Urine	Faeces	(g.)	(%)		Urine	Faeces	(g.)	(%)	Initial	Final			
						Exp. 1										
Bread 1: white+low level *Creta praeparata*	233·75	0·1099	0·0080	0·0245	0·0774	70·5	0·2606	0·1033	0·0676	0·0897	34·2	49·3	71·7	5·6	13·2	43·5
Bread 2: white+low level bone meal	227·21	0·1063	0·0079	0·0234	0·0750	70·6	0·2654	0·1204	0·0627	0·0823	30·8	48·3	69·0	5·9	14·7	43·7
Bread 3: wholemeal	242·84	0·1175	0·0086	0·0340	0·0749	63·4	0·8414	0·4980	0·2157	0·1277	15·0	48·0	78·0	4·3	30·6	41·7
Bread 4: white+high level *Creta praeparata*	235·37	0·2245	0·0080	0·0698	0·1467	65·2	0·2763	0·0566	0·0883	0·1314	47·3	48·4	73·2	9·6	11·8	48·3
Bread 5: white+high level bone meal	241·69	0·2299	0·0078	0·0721	0·1500	65·3	0·8531	0·4302	0·0895	0·1333	37·6	49·0	77·2	8·3	12·8	48·3
						Exp. 2										
Bread 4: white+high level *Creta praeparata*	244·15	0·2329	0·0083	0·0611	0·1635	70·3	0·2866	0·0604	0·0812	0·1450	50·6	55·3	81·7	8·9	11·0	48·0
Bread 5: white+high level bone meal	242·81	0·2311	0·0076	0·0620	0·1615	69·8	0·3550	0·1340	0·0601	0·1409	39·6	56·3	81·8	9·1	13·9	48·1
Bread 6: wholemeal+*Creta praeparata*	310·60	0·3053	0·0092	0·0903	0·2058	67·2	1·0943	0·5891	0·2829	0·2223	20·3	55·5	106·5	6·1	21·7	45·7
Bread 7: wholemeal+bone meal	310·61	0·3016	0·0097	0·0898	0·1921	63·6	1·1356	0·6337	0·2873	0·2146	18·9	54·2	103·5	6·2	23·2	45·1
						Exp. 3										
Dried skim milk	298·99	0·4981	0·0065	0·0087	0·4829	96·9	0·7310	0·3531	0·0613	0·3166	43·3	76·3	141·5	7·8	11·5	—
Creta praeparata	296·37	0·4970	0·0061	0·0345	0·4564	91·8	0·7107	0·3474	0·0570	0·3063	45·4	76·5	140·5	8·4	11·9	—
Bone meal	298·94	0·4855	0·0066	0·0541	0·4248	87·5	0·7091	0·3364	0·0839	0·2888	40·6	76·0	143·8	7·5	10·9	—

* Six animals were used for all breads, with the exception of breads 4 and 5 in Exp. 1, for which only five animals were available.

Table 4. *Statistical treatment* of differences in percentage* Ca *retentions in the three experiments*

Difference tested	Numerical difference	Standard error of the mean	P†	
		Exp. 1		
Breads 1, 2	−0·1	±2·8	1 : 1	N.S.
Breads 1, 3	+7·0	±2·1	1 : 47	S.
Breads 2, 3	+7·2	±2·2	1 : 47	S.
Breads 4, 5	−0·1	±2·4	1 : 1	N.S.
		Exp. 2		
Breads 4, 5	+0·5	±3·8	1 : 1	N.S.
Breads 6, 7	+3·6	±2·1	1 : 7	N.S.
Breads 4, 6	+3·1	±2·5	1 : 3	N.S.
Breads 4, 7	+6·7	±3·0	1 : 13	N.S.
Breads 5, 6	+2·6	±3·5	1 : 2	N.S.
Breads 5, 7	+6·2	±2·2	1 : 27	S.
		Exp. 3		
Milk, *Creta praeparata*	+5·1	±0·7	1 : 1086	S.
Milk, bone meal	+9·4	±0·8	1 : 14286	S.
Creta praeparata, bone meal	+4·3	±0·8	1 : 333	S.

N.S. = not significant; S. = significant.

* All comparisons were made between litter-mates using the paired t-test of 'Student' (1908, 1925).

† P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

of the availability of the Ca of *Creta praeparata* and bone meal when added to the bread.

When the Ca intake was increased (breads 4 and 5) the retention also rose markedly. As a matter of fact it almost kept pace with the increased supply. The growth of the rats was slow, of the same order as that observed by us before with rats receiving bread supplemented only with $CaCO_3$ (Henry *et al.* 1941), and their intake of Ca/g. gain in weight outstripped the 'normal' requirement over this weight range (7·5 mg./g.) as defined by Sherman & Macleod (1925). Hence these animals were probably ingesting more Ca than they were able to utilize, and there was thus a rise in their faecal excretion of Ca and a slight lowering of the percentage retention of this element.

The findings with breads 4 and 5 in the second experiment are very similar to those reported in the first experiment. Ca was again equally well retained from both breads; the absolute and percentage retention was somewhat higher than in Exp. 1. This is accounted for by a lower excretion of Ca in the faeces.

All five diets supplied relatively more P than Ca and the amounts of P consumed were well above the 'norm', 6 mg./g. gain in weight, of Sherman & Quinn (1926). The retentions from breads 1 and 2 were substantially the same, about 80–90 mg., but some 130 mg. were retained from the wholemeal bread. Rats receiving this bread made slightly

better growth than those on the supplemented white breads, and part of this excess P was certainly used to supply the needs of the greater weight of their soft tissues. The depositions of Ca were sensibly the same in all three groups, hence the amounts of P used for bone must have varied but little, and all the extra P must have been retained in the soft tissues. There is no doubt that the higher P retention with wholemeal bread represented a greater saturation of tissues than with breads 1 and 2.

Thus it can be calculated from the data of Table 3, on the assumption that 99% of the Ca is stored in the bones and that the Ca/P ratio of the normal rat bone is 2·3:1 (Brooke, Smith & Smith, 1934; Kramer & Shear, 1928), that rats receiving bread 1 retained 2·6 mg. of extra-skeletal P/g. gain in weight, whereas wholemeal bread gave a retention of 3·2 mg./g. gain in weight. The reason for this increased retention of P from wholemeal bread is not clear; though the amount of P supplied was very much greater than with the white breads, there is no doubt that even the latter contained more than enough P, especially as much more was absorbed than was retained.

The animals receiving the supplemented wholemeal breads consumed some 30% more diet and Ca than their litter-mates eating the supplemented white breads; they also grew almost twice as fast, and consequently their Ca intake judged by the standard described on p. 119 was suboptimal while that of the white-bread animals was again rather above the normal. Hence a comparison of the percentage retentions of Ca from the two types of bread would be biased in favour of the wholemeal groups. Nevertheless, relatively more Ca was retained from the white breads, the difference between breads 5 and 7 being statistically significant, and it is highly probable that Ca was not quite as available from wholemeal bread as from white bread. There is also an indication that the phosphate was less satisfactory than the carbonate as an addition to wholemeal bread, though statistical confirmation is lacking.

The results of the direct comparison of the carbonate with the phosphate carried out in Exp. 3 (Tables 3, 4) point more forcibly in the same direction. The rats in this experiment grew faster than any animals in the previous two experiments and reached a higher final weight. The 'normal' Ca requirement of rats of their size is 8·8 mg./g. gain in weight (Sherman & Macleod, 1925), and their actual intakes may be taken as suboptimal. The retention of Ca from *Creta praeparata* and bone meal was 92 and 87% respectively; Table 4 shows that this difference was statistically significant. Of the milk Ca 97% was retained. This figure was statistically superior to those obtained for the mineral additions and confirms our earlier finding (Henry & Kon, 1939*a*) of the very high availability of milk Ca. The value of 87% now obtained for bone meal may be compared with the values, 96 and 92%, previously reported by us for $CaHPO_4.2H_2O$ (Henry & Kon, 1939*a*, *b*).

It should be recorded that in a recent paper Yudkin (1943) found the carbonate and the phosphate of Ca equally effective in increasing the bone-ash content of rats, when added to white flour, wheatmeal flour, or maize gluten mixture, in quantities similar to those used by us.

A comparison of the P retention from breads 1 and 4 is of interest as the diet and P intakes from both were very similar, though the Ca intakes naturally differed. The increased retention of Ca from bread 4 was, as would be expected, accompanied by additional deposition of P. However, the P retained for this purpose accounts for only 70% of the difference in retention from the two diets, and the extra-skeletal stores must have been rather better supplied from bread 4. The better retention of P was chiefly due to a diminished urinary excretion.

From the figures for the metabolism of P derived from bread 3 it is possible to gain an insight into the utilization of phytate P by the rats. Of the 0·8414 g. P supplied by this bread, 0·4075 g. was phytate P and 0·4339 g. non-phytate P (cf. Tables 1, 3). As the absorbed P amounted to 0·6257 g., it must have contained at least 0·1918 g. of phytate P, or 47% of that present in the bread. This calculation underestimates the availability of phytate P and sets a minimum figure for its utilization.

It is obvious that non-phytate P is not 100% available, and to obtain a more satisfactory estimate a correction might be introduced for the non-phytate P lost through the gut. For example, it might be assumed that the non-phytate P of brown bread is as available as that of white bread. For this calculation the very small amount of phytate P in white bread may be neglected. The intake of P from bread 1 was 0·2606 g., of which 0·0676 g. was excreted in the faeces. Taking the minimum unavoidable faecal excretion of P as 1·1 mg. daily (Henry & Kon, 1939*a*), one finds that 0·0291 g. of the bread P was not available; *pro rata* the unavailable non-phytate P of the brown bread would amount to 0·0485 g., and the absorbed phytate P would be not 0·1918 g. but 0·2403 g. or 59%.

The higher limit for the availability of phytate P may be calculated in the following way: it may be assumed that the excess/100 g. diet of faecal P derived from bread 3 over that obtained from bread 1 represents unabsorbed phytate P. The excess works out at 59·9 mg., and as the phytate P intake in 100 g. of bread 3 was 167·8 mg., 36% of the intake was excreted. The 64% hydrolyzed in the intestine would represent the maximum phytate P potentially available. Similar calculations with breads 4 and 6 yield figures for the minimum estimate of

46%, for the corrected estimate of 62% and for the amount hydrolyzed in the intestine of 66%.

In this connexion it is of interest to point out that though the excess faecal P derived from bread 3 as compared with bread 1, or from bread 6 as compared with bread 4, may well be phytate P, it cannot possibly be in the form of Ca phytate. Ca phytate contains almost equal weights of Ca and P (Krieger *et al.* 1940*b*), yet the excess faecal P/100 g. bread was 59·9 mg. for bread 3 and 57·8 mg. for bread 6, while the excesses of Ca were only 3·5 and 4·1 mg. respectively. The similarity of the values obtained for Ca and P in the two independent experiments is noteworthy.

A clearer indication of the effects of wholemeal bread on the assimilation of Ca may be gained from a consideration of the faecal excretions. It will be seen that 90–100 mg. of Ca were lost in this way from the supplemented wholemeal breads, whereas only 34 mg. were correspondingly lost from the unsupplemented wholemeal bread in Exp. 1. Assuming that in both experiments the Ca of wholemeal bread was equally available, and making allowance for unavoidable faecal losses of Ca of 0·1 mg. daily (Henry & Kon, 1939*a*), it can be calculated that with breads 6 and 7 there should be a faecal loss of 39 mg. of Ca originally present in wholemeal bread. Breads 6 and 7 supplied, however, in addition 155 and 151 mg. Ca respectively from *Creta praeparata* and bone meal. From the availability of Ca from these sources found in Exp. 3, their contribution to faecal Ca may be calculated as 11 and 18 mg. respectively. The total calculated amount of unavailable Ca derived from wholemeal bread and mineral addition amounts, therefore, to 50 mg. for bread 6 and 57 mg. for bread 7; allowing for unavoidable losses one arrives at over-all figures of 54 and 61 mg. respectively. However, the actual excretions were 90 and 100 mg. respectively and the difference represents the Ca of *Creta praeparata* or bone meal rendered unavailable by the wholemeal bread.

The superior growth of the animals receiving the wholemeal breads at both levels of Ca intake is of interest. In Exp. 1 the intake was very similar for breads 1, 2 and 3 and the calorie intake, calculated from data in the tables of McCance & Widdowson (1940), was, if anything, lower from the wholemeal bread, yet all the animals receiving the wholemeal bread grew faster than their litter-mates on the white breads. In Exp. 2 the consumption of wholemeal bread was some 28% higher and growth some 100% better than with white bread.

Extensive studies by Chick (1940, 1942) and Copping (1943) have shown the superior nutritive value, as measured on rats, of higher extraction flours. The present results may be taken to confirm and support their findings. It should be noted that in our experiments ample quantities of vitamin B_1, riboflavin, and pantothenic acid were added to all breads. The superiority of the wholemeal bread which manifested itself under these circumstances was, therefore, probably due to differences in other factors.

DISCUSSION

Viewed as a whole our experiments certainly support the findings of McCance & Widdowson (1942*a*) that breads made from higher extraction flour have a depressing effect on the assimilation of Ca. Our experiments were of a more drastic nature in that all the calories and Ca were derived from bread, while the human diet was more varied and with it only about 50% of calories was obtained from bread. Yet the effects observed by us were much milder than those noted by McCance & Widdowson. The explanation probably lies in the choice of our experimental animal. A young growing rat will, under suitable conditions, absorb nearly 100% and retain over 98% of ingested Ca (Ellis & Mitchell, 1933; Henry & Kon, 1939*a*), while children and adults utilize only some 20% (cf. Kon, 1943). Apart from that, it seems possible that the possession of an intestinal phytase enables the rat to break down phytate P more efficiently than man. Though the destruction of phytic acid in the human gut proceeds apparently to a marked extent (Rogoziński, 1910; McCance & Widdowson, 1935, 1942*a*), a large part of it may take place at a level too low for absorption to occur. In the rat the enzymic breakdown higher up in the gut may well favour absorption. In fact, our figures indicate an absorption by the rat of some 60% of the ingested phytate P as against the estimate for man of 50% by McCance & Widdowson (1942*a*). There is ample evidence in the literature, already quoted on p. 117 of this paper, that phytate P is less well utilized by the rat than other forms of this element, and that in the presence of large quantities of phytic acid the metabolism of Ca and P as measured by the deposition of bone may be impaired through shortage of available P. The evidence, however, for any marked direct depressing effect of phytic acid on the utilization of Ca by the rat, as found by Harrison & Mellanby (1939) for the dog and by McCance & Widdowson (1942*a*, *b*) for man, is almost entirely lacking. In fact Krieger *et al.* (1940*b*) report that the Ca of Ca phytate is as available to the rat as that of $CaCO_3$. We believe, however, that our observations prove that, in the rat, phytic acid exerts effects similar to those in man and the dog, though probably to a lesser degree. The depressing effect of phytate P on the utilization of Ca by the rat has also very recently been reported by Marcy (1944). He used diets containing 0·12–0·25% P and with a Ca/P ratio of 2:1.

SUMMARY

1. The retention of Ca by rats receiving diets mainly consisting of wholemeal bread or of bread made from 72% extraction flour has been studied in metabolic experiments.

2. In one experiment *Creta praeparata* or bone meal were added to the 72% extraction flour to bring the Ca content of the diet to that of the wholemeal bread diet. In another, the two substances were added to the 72% extraction flour at a rate equivalent to ½ lb. of $CaCO_3$/280 lb. sack of flour, and the wholemeal flour was similarly supplemented to bring its Ca content to the same level.

3. In both instances Ca was less well retained from the wholemeal bread diets than from the diets containing bread made from lower extraction flour, and the evidence strongly suggests that this was due to the depressing effect of the phytic acid present in wholemeal bread. These results confirm the observations on man by McCance & Widdowson (1942*a*).

4. The wholemeal bread diets promoted better growth in rats than the 72% bread diets.

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The Formation of Mono- and Di-glycerides during the Hydrolysis of Triglyceride by Pancreatic Lipase

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It has been generally assumed that triglyceride is rapidly broken down by pancreatic lipase to fatty acid and glycerol. This is a necessary assumption if it is believed that triglyceride must be completely hydrolyzed to fatty acid and glycerol before it can be absorbed from the intestinal lumen (Verzar & McDougall, 1936; Bloor, 1943). It can be shown, however, that triglyceride and fatty acid behave differently both during and after absorption. Triglyceride absorption is associated with globular dispersion of fat in the intestinal cell, with milkiness of the lacteals, with an increase of the particulate fat in the systemic blood and with subsequent deposition in the fat depots, whereas fatty acid absorption gives rise to a granular appearance in the intestinal cell, an increase of fatty material in the portal blood and deposition in the liver (Frazer, 1938, 1943*a*). It is generally agreed that trigly-

cerides are emulsified in the small intestine. An effective intestinal emulsifying system must fulfil four criteria. Emulsification must be spontaneous without violent agitation, the emulsion particle diameter should be less than 0·5μ, the emulsion must be stable for a period of at least 3 hr. and the system must be effective over a pH range of 6·0–8·0. The possible chemical factors in intestinal emulsification of fat have been studied in single, double and triple combination. The only system which satisfied the necessary conditions was the triple combination of monoglyceride/fatty acid/bile acid (Frazer, Schulman & Stewart, 1944). The object of this communication is to give evidence for the formation of mono- and di-glycerides (henceforth called lower glycerides) during pancreatic lipolysis both *in vitro* and *in vivo*. The possible significance of these lower glycerides in the absorption of fat from the intestine is discussed.

METHODS

Reagents. Olive oil (Pool; acetyl value 5, fatty acid content 0·3%) was used in these experiments to correlate them with previous biological studies.

Lipase A: Suspension (60 mg./ml.) of pancreatin (Parke Davis and Co.).

Lipase B: Suspension (60 mg./ml.) of dried pancreas (Evans, Sons, Lescher and Webb Ltd.).

Bile acids: Sodium tauroglycocholate (British Drug Houses Ltd.).

Buffer solutions: Appropriate mixtures of 0·1 M-KH_2PO_4 and 0·1 M-NaOH.

Standard procedure for hydrolysis and extraction of products. A mixture of buffer solution (5 ml.), aqueous lipase suspension (1 ml.), and bile acid solution (0·1 ml. of 0·25 M) was warmed to 37°; olive oil (1 ml.), also at body temperature, was then added. The whole was incubated at 37°, the mixture being shaken by hand every 20 min. At the end of the experimental period glacial acetic acid (1 ml.) was added, and the mixture washed into a 250 ml. conical flask with a 50% ether-ethanol mixture (65 ml.). This mixture was heated to boiling to coagulate the proteins, allowed to cool, filtered, and the solvents removed *in vacuo* at 50°. To remove traces of acetic acid 50% ethanol (25 ml.) was now added and this was again removed *in vacuo*. Dehydration was effected by repeated treatment with ethanol, and the oily residue was extracted three times with ether and the extract filtered. The ether was removed *in vacuo* leaving an oil which was further examined; the non-oily residue was analyzed for glycerol. Control experiments were carried out using this technique with glycerol, glycerol and lipase, and glycerol and olive oil without lipase. Bile acids (0·5%; the probable concentration in intestinal contents) were normally added.

Analysis of hydrolysate. The acetyl value of the oily material was determined by the method described by West, Hoagland & Curtis (1934) using acetic anhydride in pyridine. The fatty acid value was determined by titration with sodium ethoxide. The glycerol was estimated by the method of Smith & Duke (1941).

Separation of lower glycerides. Various methods of separation of the lower glycerides from the glyceride residue were attempted. Success was eventually achieved with phthalic anhydride, by a method modified from that of Coggan & Copenhaver (1939), who employed it for the separation of aliphatic alcohols. The method can be considered in three stages.

(1) Separation of glyceride residue. Digestion was allowed to proceed normally, and, at the conclusion of the experimental period, the mixture was made alkaline with 2 N-Na_2CO_3. The glyceride fraction was removed from the alkaline solution by continuous extraction with ether.

(2) Formation of phthalic acid derivative of lower glycerides. To the glyceride fraction an equimolecular proportion of phthalic anhydride was added together with anhydrous pyridine. The clear solution was heated at 105–110° for 1 hr. After cooling, water (1 l.) was added, and the whole filtered through Kieselguhr under slightly reduced pressure till a clear filtrate was obtained. The Kieselguhr was extracted with ether which was dried over anhydrous Na_2SO_4. When the solvent was removed, a clear yellow oil was obtained.

(3) Separation of phthalic acid derivatives. The yellow oil obtained above was exactly neutralized with 0·5 N-Na_2CO_3 solution and the uncombined glyceride fraction removed by ether extraction. The remaining clear aqueous solution was then acidified with 2 N-HCl and continuously extracted with ether, when a golden brown oil was finally obtained. The properties of this oil were examined and compared with an authentic phthalic derivative of monostearin. The acid value of the phthalate was estimated before and after hydrolysis, and the decomposition products were recovered quantitatively; these results were compared with the calculated theoretical values. The authentic specimen of monostearin phthalate was prepared from recrystallized monostearin (94% pure, m.p. 63–65°) by the method described above.

In vivo *lipolysis.* The animals used were young adult rats from our own breeding stock. To obtain the material from the lumen of the intestine, the animals were anaesthetized with ether, and the olive oil was injected intragastrically. For experimental periods of more than 6 hr. a ligature was applied to the ileo-caecal junction. At the end of the experiment the intestinal contents were removed and the material from 12 rats was pooled to provide sufficient for satisfactory analysis.

RESULTS

(1) *The products of pancreatic lipolysis after periods of* 2–48 *hr.*

In vitro lipolysis was carried out as described above for varying time intervals. The fatty acid content of the fatty material was estimated and the acetyl value of the glyceride residue was determined. The non-fatty residue was analyzed for free glycerol (Table 1).

(2) *Destruction of glycerol during lipolysis*

It has been claimed (Bull, 1937) that glycerol is destroyed in the course of lipolysis. Control experiments were therefore made to determine whether the lack of glycerol in the non-fatty residue was accounted for by non-liberation from the glycerides

Table 1. *The products of pancreatic lipolysis after periods of* 2–48 *hr.*

(The results for each hour represent the average of three experiments. Bile acid 0·5%, pH 7·0. A blank mixture without oil was analyzed simultaneously in each experiment as a check on the oxidizable substances present.)

Time (hr.)	0	2	3	4	5	9	12	24	48
Total substrate (mg.)	850	843	848	819	806	804	797	837	863
Total fatty acid (mg.)	2·5	201	251	261	270	308	322	432	486
% lipolysis	0·3	24·2	29·7	31·6	33·5	38·4	40·8	51·1	56·2
Acetyl value (mg. KOH to neutralize acetic acid liberated on saponification of 1 g. of acetylated product)	5·0	47·2	58·0	60·8	64·0	67·7	74·5	77·6	66·7
% glyceride residue as monoglycerides* (calc. from fatty acid value)	—	19·9	26·6	28·8	31·7	39·4	43·3	68·6	94·0
% glyceride residue as monoglycerides* (calc. from acetyl value)	—	19·9	26·6	28·8	31·0	35·6	40·6	53·6	55·0
Free glycerol (mg.)	—	0·0	0·0	0·0	1·8	4·2	6·3	12·0	6·0
Expected glycerol (mg.) if hydrolysis complete	—	21·9	27·7	29·0	29·7	33·5	35·3	47·8	53·0

* The lower glycerides are calculated as monoglycerides in this and subsequent tables, although in most cases they consist of a mixture of di- and monoglycerides.

or by its subsequent destruction. It will be seen from Table 2 that glycerol shows no significant destruction during the first 5 hr. of lipolysis and destruction is not marked until after 24 hr.

Table 2. *Destruction of glycerol during lipolysis at* pH 7·0

	Composition of substrate mixture				
	Glycerol only	Glycerol with oil	Lipase, bile acid (0·5%), glycerol		
Time in hr.	0	0	2	24	48
Glycerol taken (mg.)	20·3	57·2	20·3	20·3	20·3
Glycerol found (mg.)	20·4	54·5	19·7	17·3	2·9

(3) *Relationship of potency of lipase to lower glyceride formation*

A. *Effect of varying* pH. The effect of pH, which is known to influence lipolytic activity, was tested upon the formation of lower glycerides. It will be seen from Table 3 that the amount of lower glycerides formed in relation to fatty acid liberated is not affected over the pH range of 6–10·5.

B. *Effect of varying bile acid concentration.* Table 4 shows the results obtained. Increase of bile acids from 0·5% upwards did not result in any decrease of lower glyceride formation.

C. *Effect of varying enzyme/substrate ratio.* The third possible factor which might account for partial instead of complete hydrolysis of triglyceride was

Table 3. *Effect of varying* pH *on lipolysis*

(Substrate mixture contained 0·5% bile acid.)

pH	6·0	7·0	7·5	8·0	10·5
Time (min.)	120	120	120	120	120
Total substrate (mg.)	869	813	772	841	820
Total fatty acid (mg.)	184	201	211	221	185
% lipolysis	22·4	24·2	27·4	26·3	22·6
Acetyl value	44·3	47·2	54·2	52·1	44·8
% glyceride residue as monoglycerides (calc. from fatty acid value)	18·1	19·9	23·7	22·4	18·4
% glyceride residue as monoglycerides (calc. from acetyl value)	18·1	19·9	23·7	22·4	18·4
Free glycerol (mg.)	0·0	0·0	0·0	0·0	0·0
Expected glycerol (mg.) if complete hydrolysis	20·0	21·9	23·0	24·0	20·0

Table 4. *Effect of varying bile acid concentration at* pH 7·5 *on lipolysis*

Bile acids added as % of total solution ...	0	0·2	0·5	1	2	4
Time (min.)	120	120	120	120	120	120
Total substrate (mg.)	771	717	772	753	723	749
Total fatty acid (mg.)	21·8	150	211	222	209	251
% lipolysis	2·8	20·9	27·4	29·5	28·9	33·5
Acetyl value	5·6	41·5	54·2	58·5	57·4	66·5
% glyceride residue as monoglycerides (calc. from fatty acid value)	1·9	16·6	23·7	26·4	25·6	31·8
% glyceride residue as monoglycerides (calc. from acetyl value)	1·9	16·6	23·7	26·4	25·6	31·8
Free glycerol (mg.)	0·0	0·0	0·0	0·0	—*	—*
Expected glycerol (mg.) if complete hydrolysis	2·38	16·3	23·0	24·1	22·7	27·3

* Results not reliable owing to frothing during concentration.

the amount of enzyme present. This was increased in two ways—by addition of more lipase solution, and by increasing the amount of pancreatic extract in the lipase solution. The results are shown in Table 5. It will be seen that increases up to fourfold did not result in any diminution in the proportion of lower glycerides formed.

(4) *Separation of lower glycerides from glyceride residue*

The glyceride residue was fractionated by the method described above. The results are shown in Table 6.

Table 5. *Effect of varying enzyme/substrate ratio on lipolysis*

(i) Effect of addition of more lipase (60 mg. pancreatin/ml.) at pH 7·0

	Lipase suspension added (ml.)			
	1	2	4	8
Time (min.)	120	120	120	120
Total substrate (mg.)	813	794	811	831
Total fatty acid (mg.)	201	194	213	194
% lipolysis	24·2	24·4	26·3	23·4
Acetyl value	47·2	48·5	52·2	46·4
% glyceride residue as monoglycerides (calc. from fatty acid value)	19·9	20·4	21·5	19·2
% glyceride residue as monoglycerides (calc. from acetyl value)	19·9	20·4	21·5	19·2
Free glycerol (mg.)	0·0	0·0	0·0	0·0
Expected glycerol (mg.) if complete hydrolysis	21·9	21·1	23·2	21·1

(ii) Effect of increasing the concentration of pancreatin at pH 7·5

	Concentration of lipase (pancreatin (mg.) contained in 1 ml. of suspension)				
	60	120	120	240	240
Time (min.)	120	120	120	120	120
Substrate (mg.)	772	804	825	812	797
Total fatty acid (mg.)	211	204	233	213	211
% lipolysis	27·4	25·4	28·2	26·2	26·5
Acetyl value	54·2	50·1	56·0	52·1	52·6
% glyceride residue as monoglycerides (calc. from fatty acid value)	23·7	21·4	24·8	22·5	22·7
% glyceride residue as monoglycerides (calc. from acetyl value)	23·7	21·4	24·8	22·5	22·7
Free glycerol (mg.)	0·0	0·0	0·0	0·0	0·0
Expected glycerol (mg.) if complete hydrolysis	23·0	22·2	25·3	23·2	23·0

Table 6. *Separation of lower glycerides from glyceride residue*

	Properties of lower glycerides				
	Authentic monostearin diphthalate			Diolein mono-phthalate	
	Actual	Calc.	Specimen I	(calc.)	Specimen II
Acid value (mg. KOH equiv. to 1 g.):					
(*a*) Before hydrolysis	172	171	148	73	77
(*b*) After hydrolysis	399	428	346	292	297
Decomposition of 1 g.:					
(*a*) Phthalic acid (mg.)	448	508	404	216	213
(*b*) Fatty acid (mg.)	448	434	591	734	679
(*c*) Glycerol (mg.)	112	141	—	120	133

Solubility. All these phthalic acid derivatives are soluble in ether, chloroform, benzene, acetone and ethanol, but insoluble in water.

Melting-point. Monostearin diphthalate is an oil at room temperature.

Authentic preparation. Condensation of monostearin with phthalic anhydride did not give a quantitative yield of diphthalate, but this was separated by solution of its sodium salt in cold water.

Specimen I is a mixture of approximately two-thirds monoglyceride diphthalate and one-third diglyceride monophthalate.

Specimen II is almost pure diglyceride monophthalate.

(5) *Formation of lower glycerides* in vivo

Material obtained from 12 rats was analyzed. Each animal was given 2 ml. olive oil intragastrically and killed 16 hr. later. The intestinal contents were found to contain 384 mg. of fatty acid, representing 30% lipolysis. The acetyl value of the glyceride residue was 70, equivalent to 31·8% of monoglyceride.

DISCUSSION

There are at least four sources of lipase in the alimentary tract: gastric, pancreatic and intestinal secretions, and lipoclastic bacteria. Of these, the most important for absorption of fat is the pancreatic lipase. Pancreatic secretion is brought about by two mechanisms, a reflex flow initially which is rich in ferments, and a more prolonged hormonic flow with a poor enzyme content (J. Mellanby, 1925). With moderate amounts of fat the peak lipaemia in the systemic blood occurs 2½–3 hr. after ingestion (Frazer & Stewart, 1937), and the blood fat has returned to the normal level within 4½ hr. Larger amounts of fat may cause a delay in the maximum lipaemia for periods up to 6 hr. after ingestion, mainly due to increase in stomach emptying time. Therefore, the first 5 hr. of pancreatic lipolysis is the important period biologically.

It is clear from the experiments described that the products of pancreatic lipolysis during the first 5 hr. are fatty acid and lower glycerides. The increase in the acetyl value of the glyceride fraction and the absence of free glycerol in the non-fatty residue can hardly be interpreted otherwise. This conclusion is further strengthened by the separation of the phthalic acid derivatives of the lower glycerides, the properties of which compare reasonably well with those of an authentic preparation. The correlation between the actual and expected figures in this experiment is reasonably close when it is considered that the preparations studied have been derived from a natural mixed triglyceride. The formation of lower glycerides has been reported previously for hydrolysis by acid and by ricinus lipase (Armstrong & Gosney, 1914; Grün & Corelli, 1912), but so far as we are aware it has not been previously reported during pancreatic lipolysis and certainly has not been stressed. The absence of free glycerol has been explained (Bull, 1937) as being due to glycerol destruction, but this is not a significant factor for the first 5 hr.

It seemed possible that the formation of lower glycerides might be an expression of low lipolytic potency in our pancreatic preparations. But no significant differences in the proportion of lower glycerides formed or in the liberation of glycerol during the first 5 hr. of hydrolysis could be demonstrated when different sources of lipase were used, when the pH was varied between 6 and 10·5, when the bile concentration was altered from 0·2 to 4%, or when the lipase present was increased up to fourfold.

When the amount of lipase was increased, there was no marked rise in the percentage lipolysis. This is in accord with the findings of Willstätter, Waldschmidt-Leitz & Memmen (1922), who showed that the addition of lipase beyond a certain point causes no further enhancement of hydrolysis. The amount of lipase used in our experiments is well above this limiting value. The inability to cause increased lipolysis *in vitro* appears to be in contradiction to the observation made in human subjects and animals that the addition of lipase after ingestion of fat causes a depression of the post-absorptive systemic lipaemia (Frazer, 1943*b*). The explanation of this apparent anomaly may be that the lipase which is normally secreted into the intestine is below the limiting value described by Willstätter *et al.* (1922). Alternatively, the difference may be due to the time relationships of the addition of lipase to the incidence of emulsification, a possible explanation of the failure of Balls, Matlack & Tucker (1937) to demonstrate monostearin in their investigation of tristearin hydrolysis. These, and other possibilities, are under investigation.

It has been frequently suggested that lipolysis may occur more rapidly in the intestine than *in vitro* because the end-products are removed. It is doubtful, however, whether the Law of Mass Action is applicable in this way to the hydrolysis of fat suspended in an aqueous medium, and there is little doubt that the slowing up of hydrolysis *in vitro* is not due to the establishment of a simple equilibrium due to the accumulation of end-products. However, to ensure that complete hydrolysis does not occur when lipase is acting in the intestinal lumen, the intestinal contents of a group of animals were examined. The content of lower glycerides in this material was comparable to that found in our other experiments.

The formation of lower glycerides during pancreatic lipolysis is probably essential for the emulsification of fat in the small intestine. Up to the present time no satisfactory explanation of this phenomenon has been put forward. It has been attributed to the bile acids, but it can be shown that these substances, if freed from soaps, do not lower the interfacial tension between oil and water sufficiently to allow more than crude emulsification, with particles 2–10 μ in diameter and of low stability. Another emulsifier suggested is soap, which will lower the interfacial tension to suitable levels provided that the reaction is alkaline. There is no doubt, however, that the pH of the contents of the small intestine may range from pH 6·3 to 8·0, and the usual reaction is pH 6·5 (Kostyal, 1926; Robinson, 1935). Soap is quite ineffective as an emulsi-

fying agent below pH 7·5, especially in the presence of small quantities of calcium salts. The triple combination of monoglyceride/fatty acid/bile acid is an effective system under the conditions found in the intestine (Frazer *et al.* 1944). If this is the normal emulsifying system, then one of the functions of lipolysis is to provide two of the three essential constituents for emulsification.

Is fine emulsification provided to enable particulate absorption to occur, or is it simply a preliminary stage to more extensive lipolysis? Since finely dispersed paraffin, upon which lipase can make no impression, is rapidly absorbed from the small intestine (Frazer *et al.* 1944), it seems reasonable to suppose that finely dispersed triglyceride fat can be similarly absorbed without further hydrolysis. Furthermore, since no free glycerol is demonstrable during the first 5 hr. of pancreatic lipolysis, and 95% of ingested fat disappears from the intestinal lumen within this time, the substances absorbed can only be fatty acids and tri-, di- or monoglycerides. The essential point of the Lipolytic Hypothesis of fat absorption is the changing of glycerides into water-soluble components with the assistance of the hydrotropic action of the bile acids. The lower glycerides of the long-chain fatty acids are insoluble in water, and in the absence of any evidence of hydrotropes which act on monoglycerides, it must be assumed that these substances are absorbed in an insoluble form; probably by the same mechanism as triglycerides. It is suggested that the Partition Hypothesis of fat absorption provides a more satisfactory explanation of the available facts so far as the absorption of olive oil is concerned.

Fine emulsions of oil in water may flocculate in the presence of protein. This phenomenon can be shown to be dependent upon the nature of the film at the oil/water interface, the type of protein and the pH of the continuous phase (Elkes, Frazer, Schulman & Stewart, 1945). The flocculation reactions of the emulsion particle in the intestinal lumen are those of a negatively charged emulsion, and these globules are unaffected by *Cl. welchii* type A toxin lecithinase (Macfarlane & Knight, 1941). It can also be shown by analyses that there is no phospholipid in this fatty material. On the other hand, the fat particles found in the blood at the height of fat absorption give flocculation reactions similar to those obtained with a phospholipid stabilized emulsion, and *Cl. welchii* lecithinase causes a loss of stability of these oil globules (Elkes & Frazer, 1944). It is suggested that the fat particle is coated with phospholipid in the course of absorption, probably in the intestinal cell. It is likely that this phospholipid is formed at the oil/water interface, and it is tentatively suggested that the lower glyceride which is concentrated at this interface may form the basis for phospholipid synthesis.

SUMMARY

1. During the first 5 hr. of pancreatic lipolysis of olive oil no free glycerol can be demonstrated and the acetyl value of the glyceride fraction rises from 5 to 64.

2. No significant glycerol destruction can be demonstrated during the first 5 hr. of pancreatic lipolysis.

3. The formation of lower glycerides is not a result of low potency of the lipase.

4. The lower glycerides can be separated from the glyceride residue by condensation with phthalic anhydride.

5. Material recovered from the intestines of a group of 12 rats shows a proportion of lower glycerides comparable to the *in vitro* experiments.

6. It is concluded that fatty acids and di- and monoglycerides are the only products of hydrolysis of olive oil by pancreatic lipase during the first 5 hr. of digestion.

7. An emulsifying system for fat which is effective under the conditions prevailing in the intestine is the triple combination fatty acid/bile acid/lower glyceride. One of the functions of lipolysis would appear to be the formation of two of the three essential factors for emulsification.

8. The partial hydrolysis of glycerides, demonstrable during the first 5 hr. of lipolysis, is in accord with the Partition Hypothesis of fat absorption, but not with the Lipolytic Hypothesis.

9. It is suggested that lower glycerides may form the basis for the formation of phospholipid at the oil/water interface in the intestinal cell.

We wish to express our indebtedness to Prof. H. P. Gilding and Dr W. V. Thorpe for facilities provided in the Biochemistry Department here, to Prof. T. P. Hilditch, F.R.S., for helpful criticism and advice, and to Mr Gormley, of John Wyeth and Co., for a supply of monoglyceride. We also wish to thank the Sir Halley Stewart Trust for their financial assistance.

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The Thermal Destruction of Vitamin B_1

1. THE INFLUENCE OF BUFFER SALTS ON THE RATE OF DESTRUCTION OF ANEURIN AT 100°

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(*Received* 3 *January* 1945)

The rate of destruction of aneurin in boiling solutions at various pH values has been described by Farrer (1941*a*). It was realized that the work done was, in effect, the study of a first order reaction, but a formal mass-action study was not published. Traill (1941) subsequently pointed out that if log k (where k is the velocity coefficient) is plotted against pH (also a logarithmic function), the curve shown in Fig. 1 results. The relation is linear until pH 7 is reached, after which the curve rises more steeply. Further work on this problem was not possible until recently. A reference to some work by Beadle, Greenwood & Kraybill (1943), who claim that aneurin is destroyed at different rates in different buffer solutions, focused attention on the fact that in the work previously published two buffer solutions were used, the McIlvaine phosphate-citric acid buffer for pH 3, 4, 5 and 6, and the Kolthoff phosphate-borate buffer for pH 7, 8 and 9. Booth (1943), too, using phosphate and phthalate buffers, noted rates of destruction smaller than those described earlier (Farrer, 1941*a*), and suggested that the buffer used in that work contained some impurity which catalyzed the destruction of aneurin. This was not so, and it was immediately realized that the change in slope of the curve shown in Fig. 1 could be due to the occurrence of two different straight lines, one for each buffer solution. This explanation of the change of slope has been proved correct by further experimental work, and it has been found that other buffer solutions also show a linear relation between pH and log k. Furthermore, it has been found that the slope of the curve obtained by plotting pH against log k varies with the buffer solution used and with the predominating ions present.

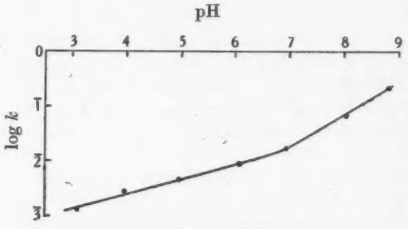

Fig. 1. pH/log k curve for the pH values studied earlier (Farrer, 1941*a*).

EXPERIMENTAL

The method of attack was the same as that previously reported. 25 ml. of aneurin stock solution were added to 200 ml. of the buffer solution and the pH precisely determined. The solution was then boiled under reflux, and samples were withdrawn at regular intervals, cooled as rapidly as possible, and the aneurin content determined by the Jansen thiochrome method.

Aneurin stock solution. Several different solutions were used throughout this work, but all were made by dissolving about 10 mg. of pure vitamin B_1 (obtained from Bayer Pharma Proprietary Ltd., Sydney) in 250 ml. of water distilled in glass. As previously reported, two or three drops of HCl added to these stock solutions render them stable for at least 3 months.

Buffer solutions. The following solutions were used: (*a*) citric acid, 0·1 M; (*b*) disodium hydrogen phosphate, 0·2 M; (*c*) potassium dihydrogen phosphate, 0·1 M; (*d*) sodium diborate, 0·05 M; (*e*) succinic acid, 0·05 M; (*f*) sodium dihydrogen phosphate, M/15; (*g*) disodium hydrogen phosphate, M/15. All solutions were made from Analar chemicals and distilled water. The McIlvaine series was made from solutions (*a*) and (*b*); the Kolthoff series from (*c*) and (*d*) for phosphate-borate, and from (*d*) and (*e*) for borate-succinic acid; and the Sorensen series from (*f*) and (*g*).

Analytical method. The method of determination of aneurin and the apparatus employed were the same as were used previously, and have been described elsewhere (Farrer, 1941*b*). Only one modification was introduced. Whereas in the previous work the *n*-butanol phase was dried over anhydrous Na_2SO_4, the modification of Wang & Harris (1939) was now employed. After the separation of the phases, 10 ml. of the *n*-butanol layer were pipetted into a clean dry Erlenmeyer flask (50 ml.), and clarified by the addition of exactly 2 ml. of ethanol. The fluorescence could then be read immediately. This modification permitted a great saving in time.

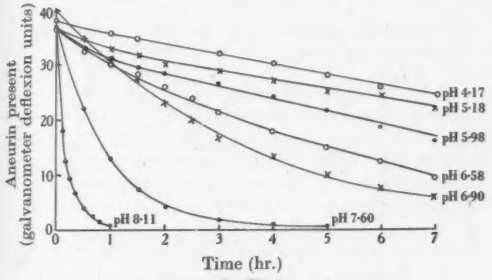

Fig. 2. Destruction of aneurin in boiling Kolthoff succinic acid-borate buffer.

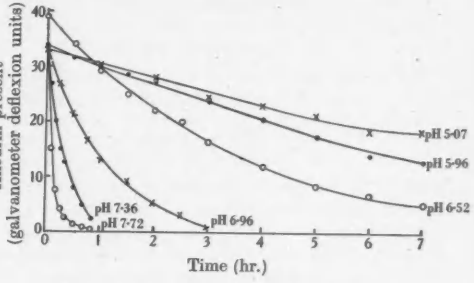

Fig. 3. Destruction of aneurin in boiling Sorensen phosphate buffer.

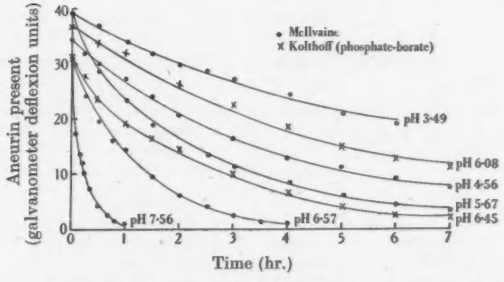

Fig. 4. Destruction of aneurin in boiling McIlvaine citric acid-phosphate, and Kolthoff phosphate-borate buffers.

Experimental procedure

(*a*) pH 3–7·5. 25 ml. of aneurin stock solution were added to 200 ml. of the desired buffer solution and the exact pH was determined by means of a Cambridge portable pH meter. Both glass and quinhydrone electrodes were used in this work, and comparisons always gave good agreement. An accuracy of ± 0·02 pH units is claimed. At the conclusion of each experiment, the pH was again determined on the solution used, and in almost every case any change in pH was within experimental error.

The solution was boiled in a 300 ml. Erlenmeyer flask connected to a Davies condenser by means of a ground-glass joint. Samples were withdrawn at given time intervals by means of a pipette, transferred to a 50 ml. Erlenmeyer flask, and cooled rapidly in running water. 5 ml. quantities were used for aneurin determinations. The ground-glass apparatus was preferred, even though the taking of samples was more cumbersome than in the case of the wash-bottle arrangement used previously and for the solutions of higher pH in this work.

(*b*) pH 7·5–8·3. The procedure previously described was used. In each case 25 ml. of aneurin stock solution were added from a pipette to a known volume of the boiling buffer solution, and a stop-watch was started when half had run in. The wash-bottle arrangement carried in a cork stopper was used, and samples were blown into 50 ml. Erlenmeyer flasks when the exact time had elapsed. Each sample was quickly cooled, and oxidized immediately with ferricyanide solution.

RESULTS

As in the earlier work, results have been expressed graphically (Figs. 2–4) by plotting galvanometer readings of the fluorimeter against time. Those obtained for the four McIlvaine and the three Kolthoff phosphate-borate buffer solutions used in the earlier

work are not repeated in this paper, but those for the intermediate pH values with these buffers are grouped together in Fig. 4. Calculations based on the earlier results are included in this work. To avoid confusion, four curves have been omitted from the graphs: the McIlvaine buffer curve at pH 8·07, which is almost the same as the Kolthoff borate-succinic acid curve at pH 8·11; the Kolthoff phosphate-borate curves for pH 7·56 and 8·32, which are very similar to the Sorensen phosphate curves pH 7·36 and 7·72 respectively; and the Sorensen phosphate curve at pH 5·50, which is virtually coincident with the same buffer curve at pH 5·07.

The barometer reading was noted for each experiment, and from the atmospheric pressure and the boiling-point elevation of water the actual boiling-point of each solution has been calculated.

Even in the case of the greatest deviation, the error was only $\pm 0{\cdot}25°$, and in most cases was very much less, so that temperature variations may be neglected and the temperature regarded as constant at 100°.

Interpretation of results

For a monomolecular reaction, the velocity coefficient, k, is given by the mass-action equation

$$k = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x},$$

where t is the time expressed in convenient units (usually minutes), a is the initial concentration, and x is the amount which has undergone change in time, t; $(a-x)$ is thus the concentration remaining after time, t, and at any given time is directly proportional to the galvanometer deflexion of the fluorimeter. The initial concentration, a, for each experiment is obviously given by the deflexion when $t=0$.

For each buffer solution, time, t, has been plotted against $\log_{10}(a-x)$, and a straight line has been obtained (Fig. 5). In many cases the reaction velocity was greater at first than the steady value ultimately reached. This was particularly noticeable at pH values greater than 7·0, and with most of the McIlvaine (phosphate-citric acid) buffer solutions.

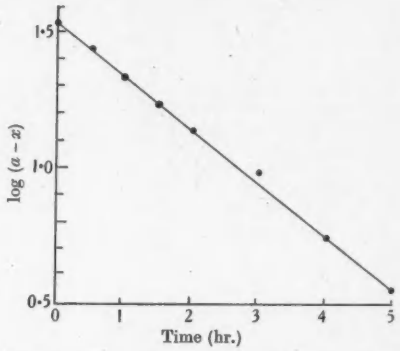

Fig. 5. Typical monomolecular reaction curve obtained for destruction of aneurin in boiling buffer solutions.

Nearly every Kolthoff buffer solution of pH 7·0 or less gave a steady velocity coefficient over the whole curve. In one or two cases, e.g. Kolthoff borate-succinic acid buffer pH 7·60, there was a change of slope at the end of the experiment, but this could be readily accounted for by experimental error in reading the feeble fluorescence remaining, small variations in the galvanometer readings causing large differences in $\log(a-x)$. The time required for the reaction to settle down was never more than an hour and generally was much less.

The steady straight line in each case was used to determine $\log(a-x)$ at different time intervals, and from these figures k has been calculated. The values obtained for k and $\log k$ are tabulated (see Table 1).

Table 1. *Values of the velocity coefficient for the thermal destruction of aneurin obtained at different* pH*'s with different buffer mixtures*

Buffer							
McIlvaine (phosphate-citric acid)		Kolthoff (phosphate-borate)		Kolthoff (borate-succinic acid)		Sorensen (phosphate)	
pH	k*	pH	k*	pH	k*	pH	k*
3·10†	0·00128	6·08	0·00288	4·17	0·000981	5·07	0·00147
3·49	0·00193	6·45	0·00614	5·18	0·00108	5·50	0·00148
3·98†	0·00269	6·94†	0·0185	5·98	0·00142	5·96	0·00210
4·56	0·00376	7·56	0·0341	6·58	0·00293	6·52	0·00478
4·97†	0·00497	8·04†	0·0715	6·90	0·00448	6·96	0·0158
5·67	0·00559	8·32	0·0935	7·60	0·0185	7·36	0·0497
6·08†	0·00933	8·84†	0·222	8·11	0·0581	7·72	0·123
6·57	0·0137						
7·15	0·0249						
7·56	0·0542						
8·07	0·0606						

* k is velocity coefficient.

† Calculated from results of Farrer (1941*a*).

Log k has been plotted against pH for each buffer solution (Fig. 6), and the curves obtained approximate roughly to straight lines. The velocity coefficient is usually inversely proportional to the hydrogen-ion concentration, and the magnitude of the effect is dependent on the nature of the buffer salts present. In order to predict the behaviour of aneurin on boiling in any given solution, it is therefore necessary to know not only the pH but also the buffer salts producing that pH.

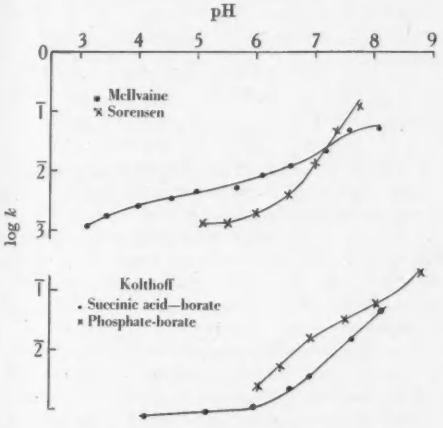

Fig. 6. pH/log k curve for destruction of aneurin in four buffer solutions

Two of the buffers, viz. the McIlvaine and the Kolthoff phosphate-borate buffers, give flattened sigmoid curves approximating to, but not coinciding with, straight lines. The curves for the Sorensen and Kolthoff succinic acid-borate buffers consist of two separate and distinct straight lines. Moreover, there is a close analogy between the course of the curve obtained by plotting pH against log k (pH/log k curve) and the course of the titration curve for the buffers employed. Points of inflexion appear at approximately the same pH values.

With the Sorensen phosphate and Kolthoff succinic acid-borate buffers, the buffering action lies in the range pH 5·9–8·0 and 3·0–5·8 respectively, where straight lines occur on the pH titration curves. Although succinic acid has two ionizable hydrogen atoms, the two dissociation constants are too close to produce any visible discontinuity in the titration curve. In the pH/log k curves there are similar straight lines in the same pH ranges. If the pH of the Sorensen buffer is moved to the acid side of the lower limit of the buffer range and that of the Kolthoff buffer to the alkaline side, the buffer curve in each case shows a change in direction, and, at approximately the same pH value, so does the pH/log k curve. In the McIlvaine and Kolthoff phosphate-borate buffers, citric acid and sodium diborate respectively are used to smooth out the curve obtained in the well-known titration of phosphoric acid with sodium hydroxide. Nevertheless, the resulting curves are not perfectly straight lines but are flattened sigmoid curves. Similarly, as noted above, the pH/log k curves for these buffers are flattened sigmoid curves which show discontinuities at approximately the same pH values as in the corresponding buffer curves.

There are, then, two similar sets of curves, and, in each, one axis is pH. In the case of the buffer titration curves, the other axis can be regarded as an indirect indication of the ionic environment of the solutions, points of inflexion corresponding with significant changes in the ionic constitution. It is therefore logical to link these changes with the discontinuities appearing at similar pH values in the pH/log k curve. It can then be stated that the rate of destruction of aneurin changes as the ionic constitution changes with rising pH. However, this change in the rate of destruction of aneurin is not solely dependent on the ionic environment, but is also conditioned by the pH in that where the change in the nature of the ions present in the solution is accompanied by a significant change in pH there is a significant change in the rate of destruction of aneurin, i.e. there is a definite change in the slope of the pH/log k curve; but where the pH is only slightly affected by the changing ionic constitution of the solution there is a correspondingly slight change in the rate at which aneurin is destroyed, i.e. there is only a slight inflexion in the pH/log k curve.

In comparing buffer titration curves at room temperature with the rate of destruction of aneurin at 100°, account must be taken of possible changes in the dissociation constants. It should therefore be pointed out, first, that the results recorded in the literature (Landolt & Börnstein, 1923) show that the change of dissociation at 100° is small for all the acids studied, and, secondly, that the points of inflexion on the pH/log k curves do not coincide exactly with those on the buffer titration curves at room temperature.

The curves published by Booth (1943) have been analyzed according to the method described in this paper. Since the actual figures were not recorded, each point on the curves was transferred to graph paper by means of a pair of dividers and the values obtained were taken as $(a-x)$. Log $(a-x)$ was then plotted against time, and k and log k were calculated from the straight lines obtained. Log k for pH 7·2 and 7·4 could not be calculated accurately enough, as there were too few points available. In Fig. 7 the pH/log k curve for Booth's phosphate buffer is compared with that for phosphate buffer

obtained in this work. The agreement is good when one considers the method by which $(a-x)$ was obtained, and also that Booth worked with 0·2M-potassium phosphate, whereas the work recorded in this paper was carried out with M/15 sodium phosphate.

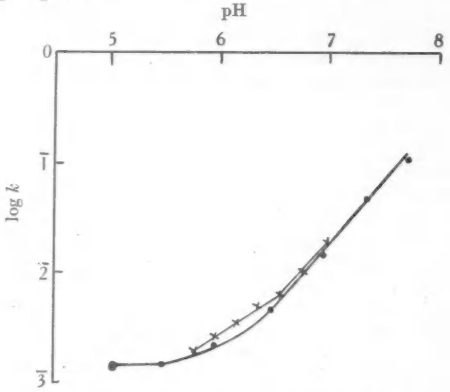

Fig. 7. pH/log k curves for phosphate buffer. (×) calculated from Booth's results. (•) results obtained in this work.

In a similar manner the values of k and log k have been calculated for the phthalate buffer (0·2M-potassium phthalate) and for the phosphate-phthalate mixture used by Booth (see Table 2).

If pH/log k curves are plotted for these buffers it is found that the shape of the curve for the phthalate buffer closely follows that for the buffer curve for phthalic acid, and that the curve for the phosphate-phthalate mixture also confirms the suggestions made herein.

In the light of the data presented in this paper, it is apparent that the explanation of the differences in the rates of destruction reported lies in the varying effect of the different ions present in the buffer solutions used by the various investigators.

Table 2. *Velocity coefficients for the thermal destruction of aneurin calculated from Booth's results*

pH	k*	pH	k*
	Phthalate buffer		
2·4	0·00076	5·2	0·0035
3·6	0·0015	5·6	0·0044
4·0	0·0022	6·0	0·0063
4·4	0·0024	6·2	0·0095
4·8	0·0033		
	Phosphate-phthalate buffer		
5·4	0·0022	6·4	0·0065
5·8	0·0031	6·8	0·0126

* k is velocity coefficient.

SUMMARY

1. The destruction of aneurin in boiling solution has been followed in various buffer solutions at pH values ranging from 3·0 to 8·0.

2. The methods used have been previously described, and the buffers studied were the Sorensen phosphate, the McIlvaine phosphate-citric acid, and the Kolthoff phosphate-borate and succinic acid-borate.

3. In general, it has been found that there is, for any given buffer, a linear relationship between the reaction velocity and the hydrogen-ion concentration, which can best be expressed by plotting pH against log k, where k is the velocity coefficient.

4. This relationship is different for each buffer, and the slope of the curve changes as the ionic constitution of the solution alters. Where this alteration is accompanied by a large change in pH, there is a big change in the slope of the curve obtained by plotting pH against log k, and vice versa.

5. In all cases, the velocity of the reaction increases as the pH rises.

The author wishes to thank the Directors of the Kraft Walker Cheese Co. Proprietary Ltd. for permission to publish this paper.

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Biochemical Characterization of the Actions of Chemotherapeutic Agents

3. RELATIONSHIPS BETWEEN METABOLIC AND GROWTH INHIBITIONS BY PANTOTHENATE ANALOGUES: THEIR STRUCTURAL AND SPECIES SPECIFICITY

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(*Received* 4 *January* 1945)

It was reported in Part 2 (McIlwain & Hughes, 1944) that pantoyltaurine inhibited a process of inactivation of pantothenate which was brought about by haemolytic streptococci. Characteristics of the metabolic inhibition suggested it to be connected with the inhibition of growth of the organism, which was also caused by pantoyltaurine, and which was shown to be the basis of its chemotherapeutic effects (McIlwain & Hawking, 1943). Little is known either of the reactions undergone by pantothenate in living organisms, or of the types of interaction which may underlie the chemotherapeutic action. Further evidence bearing upon the relationship between the two inhibitory effects of pantoyltaurine has therefore been sought. The present investigation compares the actions (1) of a series of pantothenate analogues upon the two processes in haemolytic streptococci and (2) of pantoyltaurine upon the two processes in a series of organisms.

The effects of the compounds upon growth were expressed as the concentrations of them required to affect visible growth of the organisms in the presence of defined concentrations of pantothenate, which antagonized the growth-inhibitory effects of several of the compounds. Certain analogues have been examined in this respect previously (McIlwain, 1942; Barnett, 1944; Barnett, Dupre, Holloway & Robinson, 1944; Madinaveitia, Martin, Rose & Swain, 1944). Inactivation of pantothenate was measured by microbiological assay of the amount of this compound remaining, in solutions which initially contained pantothenate and the substances under examination, after exposure of these solutions to the organisms studied.

EXPERIMENTAL

Pantothenate assay

Only microbiological methods were available for assay of the quantities of pantothenate concerned in the present experiments, and of these methods Pelczar & Porter's (1941) use of *Proteus morganii* appeared most promising, as the organism is relatively insensitive to inhibition by pantothenate analogues which would be present in certain of the reaction mixtures. As optimal growth of this organism has not yet been described in chemically defined media (see Pelczar & Porter, 1943), and as it was desired to assay metabolic mixtures and bacterial extracts without separation of pantothenate, a complex basal medium similar to that used in certain metabolic studies with pantothenate was employed.

Assay medium. To the stock solution (20 ml.) of McIlwain & Hughes (1944) (a hydrolysate of casein prepared in the presence of titanium salts), were added: KH_2PO_4, 13·6 g.; $(NH_4)_2SO_4$, 0·5 g.; NH_4Cl, 0·5 g.; anhyd. $MgSO_4$, 20 mg.; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 10 mg.; 10^{-2}M-nicotinamide, 1 ml. The whole was boiled in about 600 ml. of water for 5 min., cooled, made to 700 ml. with water, filtered, distributed in 100 ml. portions and autoclaved to sterilize. The assay medium was prepared from this solution by adding, per 100 ml., immediately before use: a cystine-glucose supplement (10 ml.); N-NaOH (2 ml.); water (6 ml.); and an opacity-standardized suspension of *Pr. morganii* containing about 10^6 cells. The supplement was prepared by dissolving cystine (0·1 g.) and glucose (5 g.) in N-HCl (10 ml.), diluting to 50 ml., and filtering to sterilize.

Assay. A 10^{-2}M solution of Ca(+)pantothenate in phosphate buffer of pH 6·5 was filtered to sterilize and kept in sealed tubes in a refrigerator. The following dilutions were prepared from it fortnightly in the same buffer: 10^{-4}, 10^{-6}, 10^{-7} and 1·5, 1·25, 1·00, 0·75, 0·5, $0·25 \times 10^{-8}$M. Reaction mixtures containing pantothenate were sterilized by filtration at pantothenate concentrations of 10^{-5}–10^{-6}M and diluted aseptically for assay to a range of concentrations expected to be about $0·5$–$1·5 \times 10^{-8}$M. One ml. each of the series of standard solutions (15–$2·5 \times 10^{-12}$ g.-mol. of pantothenate) and three dilutions of each solution to be assayed were pipetted into $6 \times \frac{3}{4}$ in. tubes (cleaned with chromic-sulphuric acid, plugged and sterilized), 5 ml. of the already inoculated assay medium added, and the tubes incubated at 37°. Between 27 and 48 hr. after inoculation the density of growth was determined

in a Hilger photoelectric absorptiometer, using neutral filters and cells taking 0·5 ml. of solution.

Choice of conditions of assay

(*a*) *Organisms.* The growth of four strains of *Pr. morganii* with varying quantities of pantothenate was examined; the response of the two strains used in assay is shown in Fig. 1. Strain no. 2818 of the National Collection of Type Cultures (N.C.T.C.) has been obtained from the National Collection on two occasions separated by 3 years and the older specimen maintained by frequent subculture in this laboratory, but the response of the two specimens did not differ appreciably. Strain N.C.T.C. 1707 and a laboratory strain gave curves similar to that of N.C.T.C. 2818, but the laboratory strain 2 was more sensitive to pantothenate. Inocula for assays were taken from 16–18 hr. cultures on broth-agar slopes; older inocula tended to give erratic results. Prior growth of inocula in the assay medium was not advantageous. Response to pantothenate with inocula of about 10^4 cells/100 ml. was slower than with the number (*c.* 10^6) adopted. It was also less directly related to the quantity of pantothenate, and this was again the case with inocula of *c.* 10^9 cells, which, however, offered no advantage, in speed of growth, over the size of inoculum chosen.

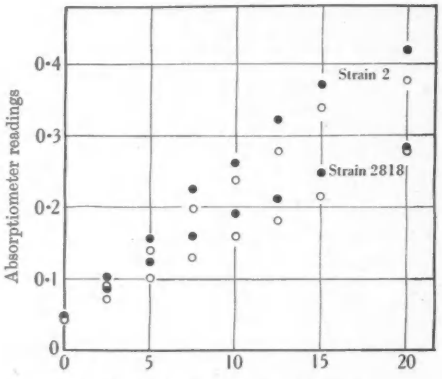

Fig. 1. Pantothenate assay by means of *Proteus morganii*. The relation between growth of two strains at 26 (○) and 48 (●) hr. after inoculation, and the quantity of pantothenate.

Readings between 27 and 48 hr. after inoculation were found equally satisfactory, but earlier or later readings less so. A little growth only took place between those times (Fig. 1). The test organism during assays formed both a diffuse growth and a deposit which easily dispersed on shaking. While the conditions of assay were being investigated, readings of samples (1·5 ml.) were taken at intervals while incubation of the bulk continued.

Dilution in the basal medium of a suspension of *Pr. morganii*, grown under the conditions of assay, and measurement of the optical densities of the resulting solutions, showed that, within the limits of accuracy of reading, densities were proportional to the quantity of organisms added. The readings were: original suspension, 0·295; 8 ml. diluted to 10 ml., 0·24; 6 ml. to 10 ml., 0·182; 4 ml. to 10 ml., 0·119; 2 ml. to 10 ml., 0·062; 1 ml. to 10 ml., 0·028.

(*b*) *Medium.* The casein hydrolysate was the only medium-constituent not chemically defined; three caseins of different origins have, however, behaved similarly. The assay medium when compared with water gave a reading of 0·056 (indicating satisfactorily little colour) which did not change after growth of the organisms in it for 2 days. The medium was highly buffered to avoid pH changes following from the active glucose metabolism of *Pr. morganii*; the change of pH over the range chosen in assay was 0·2 unit or less in 30 hr. Phosphate buffer afforded a more regular response to pantothenate than did bicarbonate; the high phosphate concentration was not deleterious to growth.

The medium was adequate to give a specific response to pantothenate. A group of nine substances which can function as growth factors for some micro-organisms (those of the group A addenda of McIlwain, 1944, not already included in the present medium) and glutathione, glutamine, and a cozymase preparation, did not increase the readings given by 10 and 20×10^{-12} g.-mol. pantothenate. Acid- and alkali-treated peptone preparations (Pelczar & Porter, 1941) and asparagine did not increase, but in some concentrations decreased, the response to pantothenate. A solution to which streptococci had been exposed under the conditions chosen for pantothenate metabolism (see below), did not affect the readings with standard pantothenate solutions. Pantoyltaurine ($0{\cdot}5 \times 10^{-6}$ g.-mol.) did not affect the assay of 5 or 30×10^{-12} g.-mol. of pantothenate.

Pantothenate inactivation

Reactions were carried out anaerobically in Warburg vessels shaken at 37° and containing, in 3·3 ml.: organisms (of dry wt. 1–10 mg.), pantothenate ($5{-}20 \times 10^{-9}$ g.-mol.), $MgSO_4$ ($0{\cdot}5 \times 10^{-6}$ g.-mol.), KH_2PO_4 (10×10^{-6} g.-mol.), $NaHCO_3$ (150×10^{-6} g.-mol.), glucose (100×10^{-6} g.-mol.), yeast adenylic acid ($0{\cdot}7 \times 10^{-6}$ g.-mol.) and the substances whose effects were being studied. Accelerating effects of the Mg, adenylic acid and phosphate had been observed in some preliminary experiments with streptococci, and these substances were therefore added

in all cases. With these additions and with the present methods of growing and harvesting the organisms, the presence of hydrolyzed casein (cf. McIlwain & Hughes, 1944) was not essential to pantothenate metabolism. The highest concentrations of pantoyltaurine which were tested were 0·075 M, and, for comparison of its effects upon different organisms, successive twofold dilutions were examined. CO_2 evolution was measured during pantothenate metabolism, but under the circumstances considered in this paper it was not found to be affected by the presence of the pantothenate analogues.

Organisms. The material used in metabolic experiments was grown aerobically from inocula of about 10^6 cells in a mixture of: the basal medium of McIlwain & Hughes (1944) (70 ml.); M-glucose (5 ml.); 0·02 M-arginine (2 ml.); 0·02 M-glutamine (2 ml.); group A of McIlwain (1944) (5 ml.) (containing one-twenty-fifth of the concentration of Fe salts there described); N-NaOH (1 ml.); 10^{-4} M-pantothenate (1 ml.); water (17 ml.).

The β-haemolytic streptococci studied included the 'Richards' strain and another group A strain; the previously employed group G strain was used in the majority of the experiments in which pantothenate analogues were compared. Two strains each of *Streptoccus faecalis*, *C. diphtheriae* (a *mitis* and a *gravis* strain) and *Pr. morganii* were examined. Organisms were collected by centrifuging and washed twice with volumes of saline one-tenth of those of the original culture.

Growth inhibition

The medium of the previous paragraph, dispensed in 10 ml. portions and containing various concentrations of pantothenate, was employed; the general conditions followed those of McIlwain (1942). Pantothenate analogues were added aseptically, as solutions which had been sterilized by filtration, in concentrations up to 10^{-2} M; if inhibitory they were examined over their range of action in twofold dilutions.

RESULTS

Structural specificity

Table 1 gives the formulae of the compounds used and summarizes their effects upon the growth of, and pantothenate inactivation by, *Streptococcus haemolyticus*. With optically active analogues, only the *dl*-forms were examined. Compounds were judged to be without inhibitory effect upon growth when no diminution of growth was seen by visual examination between 16 hr. and 7 days after inoculation of solutions containing 10^{-8} M-pantothenate and the substance under investigation in concentrations up to 2×10^{-3} M. Antagonism by pantothenate to growth inhibition by an analogue was considered absent if inhibition in the presence of 10^{-2} M-pantothenate was not distinguishable from that in the presence of 5×10^{-8} M-pantothenate. The characteristics of the compounds of Table 1 (numbers refer to that table) suggest their classification in the following three groups:

(i) *Compounds affecting neither pantothenate inactivation nor growth.* β-alanine, pantolactone and taurine (products of simple hydrolysis of pantothenate and pantoyltaurine) inhibited neither growth nor pantothenate inactivation when present in M concentrations up to 4000 times those of the concomitant pantothenate. A pantothenate homologue (6) and a substituted pantoyltaurine (7) were also without action (Table 2).

Table 1. *Pantothenate analogues, and their effects upon the growth of β-haemolytic streptococci*

Symbols used in formulae below:
P— = $CH_2OH(CH_3)_2.C.CHOH.CO$—
—*β-al* = —$NH.CH_2.CH_2.COOH$
Ts = toluene *p*-sulphonyl-

		Effect of analogues on growth	
		Inhibition	Antagonism of inhibition by pantothenate
	A. Compounds not affecting pantothenate inactivation		
(1) to (3)	Pantolactone; β-alanine; taurine	0	—
(4)	$CH_2OH.CH_2.CH_2.CO$—*β-al*	+	0
(5)	$CH_3.CH_2OH.CH_2.CH_2.CO$—*β-al*	+	0
(6)	$CH_2OH(CH_3)_2.C.CHOH.CH_2.CO$—*β-al*	0	—
(7)	$CH_2OH(CH_3)_2.C.CHOTs.CO$—$NH.CH_2.CH_2.SO_3H$	0	—
	B. Compounds inhibiting pantothenate inactivation		
(8) (pantamide)	*P*—NH_2	+	+
(9) (panthydrazide)	*P*—$NH.NH_2$	+	+
(10) (pantoyltaurine)	*P*—$NH.CH_2.CH_2.SO_3H$	+	+
(11) (pantoyltauramide)	*P*—$NH.CH_2CH_2.SO_3NH_2$	+	+
(12)	*P*—$NH.CH_2.CH_2.SO_2$–C$_6$H$_4$–NH_2	+	+
(13)	$(P$—$NH.CH_2CH_2)_2SO_2$	+	+
(14)	$(P$—$NH.CH_2.CH_2)_2S_2$	+	+

Table 2. *Pantothenate inactivation by bacteria in the presence of pantothenate analogues*

20×10^{-9} g.-mol. of pantothenate were initially present in each reaction mixture. The quantity remaining, after a period (constant with a given batch of organisms) of about 1 hr. at 37°, was determined.

Organisms: Strain and batch	Dry wt. (mg.)	Analogues and quantities ($g.\text{-mol.} \times 10^{-6}$) used. Numbers of analogues refer to Table 1	Pantothenate inactivated ($g.\text{-mol.} \times 10^{-9}$)
Strep. haemolyticus			
(group G) I	8·0	0	16·9
,, I	8·0	Pantoyltaurine, 0·4	1·1
,, I	8·0	β-Alanine, 40 *or* pantolactone, 40; *or* taurine, 40 *or* analogue (7), 4 or 40	16·7–17·2
,, II	9·5	0	18·0
,, II	9·5	Pantoyltaurine, 0·4	1·2
,, II	9·5	Analogues (4), (5), *or* (6), 4 or 40	17·6–18·1
,, III	4·4	0	4·0
,, III	4·4	Pantoyltaurine 0·012	3·7
,, III	4·4	,, 0·032	3·4
,, III	4·4	,, 0·120	1·8
,, III	4·4	,, 0·320	1·2
C. diphtheriae strain 49 (*gravis*)	3·7	0	4·1
,,	3·7	Pantoyltaurine 0·5	3·5
,,	3·7	,, 1·0	1·9
,,	3·7	,, 2·0	0·7
,,	3·7	,, 10·0	0·3

(ii) *Substances not affecting pantothenate inactivation but inhibitory to growth.* A *bisnor*deoxypantothenate (4) and an *isonor*deoxypantothenate (5) did not affect the process of pantothenate inactivation when in M concentrations 4000 times those of pantothenate, but inhibited growth. The effect of these two compounds upon growth differed from that of the group (iii) compounds (below), in the failure of pantothenate to antagonize the inhibition of growth.

(iii) *Both growth and pantothenate inactivation were inhibited* by relatively low concentrations of pantoyltaurine (10) and its amide (11); by the amide (8) and hydrazide (9) of pantoic acid and by *bis*(pantoyl-β-aminoethyl) sulphone (13) and the corresponding disulphide (14). The effects of these compounds upon pantothenate metabolism by the group G streptococcus were determined in each case by experiments of the type quoted in Table 2 (batch III). Studies, which will be reported later, of the kinetics of pantothenate inactivation have shown that experiments of this type, in which pantothenate usage is kept to about one-quarter of the total amount present, yield valid measures of the activities of the inhibitors. By interpolation between such results, the concentration of inhibitor corresponding to 50% inhibition of pantothenate inactivation was determined. These concentrations are compared in Table 3 with the concentrations of the inhibitors which affect growth. Growth was not measured quantitatively, but concentrations of the inhibitors were found which were minimal for just observable inhibition, and which completely inhibited visible growth during 2 days. Where possible these concentrations were determined in the presence of the same concentration of pantothenate as was used in metabolic experiments, but where this would have necessitated a concentration of inhibitor $> 10^{-2}$M, the pantothenate concentration used in growth was lowered. It was not necessary to decrease it below 5×10^{-3}M, which is adequate for almost optimal growth of the organism. Pantothenate antagonizes the action of pantoyltaurine and, to allow for the effects of varying pantothenate concentrations, the quantities of pantoyltaurine necessary for inhibition have been expressed in Table 3 as ratios, obtained by dividing their concentrations by that of the concomitant

Table 3. *Comparison of effects of pantothenate analogues* (8)–(14) *on bacterial growth and metabolism*

Compound (numbers refer to Table 1)	Organism	Concentration of compound (as multiple of the concentration of pantothenate present) necessary for: 50% inhibition of pantothenate inactivation (*a*)	Just observable inhibition of growth (*b*)	Prevention of visible growth for 2 days (*c*)
Pantoyltaurine (10)	(1) *Strep. haemolyticus* (group G)	5·0	40	200
,,	(2) *C. diphtheriae* strain GI (*mitis*)	13·0	100	500
,,	(3) *C. diphtheriae* strain 49 (*gravis*)	45	2,000	10,000
,,	(4) *Strep. faecalis* strain II	100	2,000	15,000
,,	(5) *Proteus morganii* strain N.C.T.C. 2818	1,450	50,000	200,000
Sulphonamide (12)	*Strep. haemolyticus* (group G)	13	160	800
Pantoyltauramide (11)	,,	25	400	2,000
Panthydrazide (9)	,,	60	800	4,000
Pantamide (8)	,,	270	4,000	20,000
Sulphone (13)	,,	350	6,600	40,000
Disulphide (14)	,,	520	8,000	40,000

pantothenate. The effect of a given ratio of pantoyltaurine and pantothenate concentrations upon growth of the present organism is only approximately constant and varies with the absolute concentrations of the compounds. The variation is, however, small in comparison with the range of values of Table 3. Approximate constancy has also been shown in the effects of a given ratio of pantoyltauramide, panthydrazide, the sulphonamide derivative (12), the sulphone (13) and disulphide (14) upon growth of the present organism and of a group A haemolytic streptococcus. Expressed as such ratios, the inhibitory powers of the compounds studied decreased from pantoyltaurine with a ratio of 40:1 needed to have an observable effect on growth, to the disulphide (14), required in a ratio of 8000:1 to produce the same effect. The seven compounds of Table 3 fell into the same order when their activity in inhibiting pantothenate inactivation was considered. The concentrations of compounds causing the different types of inhibition are not directly proportional; the ratio of the values of column (*b*) to (*a*) of Table 3 varies, with different analogues, from 5 to 19, and of column (*c*) to (*a*), from 40 to 110. The variation is, however, progressive, increasing with the increasing concentrations required of the less active compounds, and it is evident that a close connexion exists between the quantities of inhibitor required for the two effects. This is exhibited in Fig. 2*a*, and is especially striking as the concentrations required for a given growth effect vary over a 200-fold range. The curves of Fig. 2*a* are approximately linear, with slopes just greater than unity; graphic extrapolation would give positive intercepts on the ordinate. These characters reflect the lack of direct proportionality between values affecting metabolism and growth, and the ability of the compounds to inhibit metabolism (pantothenate inactivation) by 50% at concentrations which are without observable effects on growth.

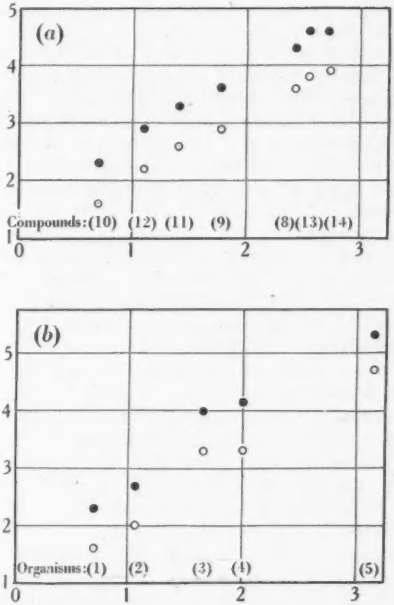

Fig. 2. Correlations between the effects of pantothenate analogues on pantothenate inactivation and growth.
(*a*) Effects of seven different analogues (numbered according to Table 1) on *Streptococcus haemolyticus*.
(*b*) The effect of pantoyltaurine on five bacterial strains (numbered according to Table 3).
Ordinates: logarithms of ratios of concentration of inhibitor to concentration of concomitant pantothenate which (o) just affect growth and (•) which inhibit growth for 2 days. *Abscissae*: logarithms of ratios of inhibitor/pantothenate which inhibits pantothenate inactivation by 50%.

Species specificity

The pantoyltaurine resistance of bacteria which synthesize pantothenate has been discussed previously (McIlwain, 1942, 1943). The present report concerns organisms requiring added pantothenate for growth, and species were chosen which required, for inhibition of their growth, widely differing minimal concentrations of pantoyltaurine. These organisms were all found to inactivate pantothenate during various metabolic processes which will be described in detail elsewhere. The effect of pantoyltaurine upon their pantothenate inactivation was compared during glycolysis, which all the organisms carried out at rates which did not greatly differ with the different organisms. Glycolysis was also a major process during the growth experiments with which the pantothenate metabolism was being compared.

The effect of pantoyltaurine upon pantothenate inactivation by a strain of *C. diphtheriae* is shown in Table 2. From such values the ratio of pantoyltaurine to pantothenate concentrations required for 50% inhibition of pantothenate inactivation was obtained for each organism. The remarks of the previous section respecting the use of such ratios apply also to the present problem, as the effects upon growth of given ratios of inhibitor/pantothenate have been found to be approximately constant in the following cases: of pantoyltaurine, pantoyltauramide, the sulphone (13) and disulphide (14) on growth of *C. diphtheriae*; of pantoyltaurine on growth of *Strep. faecalis* and *Pr. morganii*. In Table 3 the organisms are arranged in order of the sensitivity of their growth to pantoyltaurine. This is seen also to be the order of sensitivity of their

pantothenate metabolism. Again the values for the two types of inhibition are not directly proportional, but correlation between the values is shown in Fig. 2*b* and is of the same type as that of Fig. 2*a*.

A full range of comparative experiments was performed only in the case of pantoyltaurine. The species-specificity of other analogues was not the same. The sulphone (13) and disulphide (14) inhibited two strains of *C. diphtheriae* in concentrations lower than those producing comparable effects on *Strep. haemolyticus*. This order of sensitivity reverses that shown by the same organisms to pantoyltaurine. These findings do not affect the significance of the correlations of Fig. 2, but suggest that other relationships, such as that of Fig. 2*a*, might be obtained by examination of other organisms.

DISCUSSION

The action of pantoyltaurine upon bacterial growth was initially characterized as a bacteriostasis antagonized by pantothenate, and its action upon metabolism as an inhibition of pantothenate inactivation without corresponding inhibition of an energy-yielding process such as glycolysis. A connexion between the two effects of pantoyltaurine is now supported by the following evidence.

(1) Six other compounds which, like pantoyltaurine, inhibited growth and whose inhibition was antagonized by pantothenate, also inhibited pantothenate inactivation. Seven compounds whose effects on growth were without those characteristics did not affect the pantothenate inactivation. Of these seven, two are of special interest because, although they did inhibit growth, this inhibition was not antagonized by pantothenate and thus lacked the initial criterion which grouped together compounds acting in the manner of pantoyltaurine. Thus the two compounds whose action would be suggested by growth experiments to be independent of pantothenate (though the compounds, 4 and 5 of Table 1, are structurally related to pantothenate) have also been shown to be without effect on the particular reaction of pantothenate which is studied in the present experiments. The behaviour of these two compounds thus supports the general connexion between the actions of pantoyltaurine-like compounds.

(2) The seven compounds which inhibited pantothenate inactivation were required in widely different concentrations to produce a given effect, but their activities placed them in the same order as inhibitors of growth, as of inhibitors of the metabolic effect. Moreover, the concentrations required of the different compounds to produce a given metabolic and growth effect were quantitatively correlated (Fig. 2*a*).

(3) A similar correlation existed between the concentrations of pantoyltaurine which were required to inhibit growth and pantothenate inactivation with five different organisms (Fig. 2*b*).

The correlations show metabolic and growth inhibitions to be very closely connected. Thus the process of pantothenate inactivation may itself be an essential part of growth; or it may be associated with reactions which are necessary to growth. Morel (1941) regards a process which in many ways is similar to the present one as a 'wearing out' of a coenzyme during its functioning. If such a view be taken of pantothenate inactivation, it could be considered an index of pantothenate functioning, this latter being the process essential to growth. On the other hand, the pantothenate metabolism of the present non-proliferating suspensions may be a process which itself is directly coupled with growth when the presence of other nutrients permits this to occur.

Consideration of the results as they relate to the mode of action of pantoyltaurine, shows that they are consistent with the inhibition of pantothenate inactivation (or of its associated processes) being responsible for the effects of the inhibitor on growth. In the course of establishing the present correlations, a type of action similar to that of pantoyltaurine on haemolytic streptococci has been shown to be possessed by many related compounds, and upon several other bacterial species. The process of pantothenate inactivation is also seen to be widespread.

Among the compounds examined, a connexion also exists between structure and action, in that particular types of variation in the pantothenate molecule are associated with compounds capable of inhibition of pantothenate metabolism. Compounds which can be considered as derived from pantothenate through alteration in its hydroxy acid portion do not affect pantothenate metabolism, though they may affect growth by other means. The compounds inhibiting pantothenate inactivation are altered in the amino-acid portion only. These generalizations include compound (7) which is altered in both portions and does not affect pantothenate metabolism; the tosyl substituent has in this case converted the most active inhibitor of metabolism to an inactive compound. An aromatic sulphonic acid derivative does not have this effect when attached to the amino-acid portion of the molecule in analogue (12); the substituent groups are not, however, identical.

SUMMARY

1. Strains of streptococci, of *C. diphtheriae* and of *Proteus morganii* required widely different concentrations of pantoyltaurine to inhibit growth in the presence of a given concentration of pantothenate. They all inactivated added pantothenate (destroying its growth-promoting properties for the organisms) during glycolysis; pantoyltaurine inhi-

bited this inactivation. The concentrations of pantoyltaurine required for inhibition of the pantothenate metabolism by different organisms varied over a 300-fold range but were correlated with the concentrations required for inhibition of growth of the respective organisms.

2. Of 14 compounds structurally related to pantothenate, seven, which inhibited growth of haemolytic streptococci, inhibited their inactivation of pantothenate. The activities of the compounds in inhibiting the inactivation of pantothenate were correlated with their activities in inhibiting growth. Five compounds affected neither process; two compounds which inhibited growth and which were not antagonized by pantothenate did not affect pantothenate inactivation.

3. Of the compounds examined, those which inhibited pantothenate inactivation were all amides of pantoic acid; those not containing an intact and unsubstituted pantoic acid portion did not affect pantothenate inactivation.

We are greatly indebted to Drs Barnett and Robinson, and to Dr Swain, for gifts of pantothenate analogues; to the Department of Bacteriology of this University for bacterial strains, and to Miss L. Whipple for assistance during the development of the assay method.

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The Activation of Jack-bean Arginase by Cobalt, Manganese, and Iron

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The hydrolysis of arginine by preparations of jack-bean 'urease' in the presence of cobaltous ions was reported by Hellerman & Perkins (1935). Stock, Perkins & Hellerman (1938) showed that an arginase present in jack-bean extracts was strongly activated by cobalt, by manganese, and to a less extent by nickel. Cobalt produced a shift in the optimal pH from 9·0 to 7·7, and at the latter reaction the metal was necessary for activity. The present paper deals with the activation of this enzyme by cobalt, manganese, and iron, and with a preliminary investigation into the nature of the substance activated.

METHODS

Enzyme preparation. Jack-bean urease powder (Peters & Van Slyke, 1932) was used unless otherwise stated. The powder formed a stable, reproducible source of arginase and urease combined. A weighed amount of the powder was dissolved in distilled water afresh for each experiment.

Buffers. Clark & Lub's phosphate buffers and Sorensen's glycine buffers were used, allowances being made for the temperature coefficient of the latter (Clark, 1928). Clark & Lub's borate buffer could not be used, as borate inactivates urease. In a few experiments the methylglyoxaline buffers of Kirby & Neuberger (1938) were used, but their high temperature coefficient made them less suitable.

Hydrolysis. The reaction was carried out in the apparatus for the determination of urea described by Kay & Sheehan (1934). In the reaction tube were placed: 1 ml. urease solution, 5 ml. buffer, 1 ml. activating metal solution; the racks of tubes were placed in a bath at 40°; after 10 min. 1 ml. of a solution of *d*-arginine hydrochloride (2·4 g./100 ml.) was added, and the tubes containing 8 ml. total volume were stoppered and connected to the receiving tubes. The reaction mixture was stirred at intervals by drawing a gentle current of air through the apparatus. At the end of 1 or 2 hr. the tubes were cooled rapidly, and the reaction was stopped by adding 20 ml. of saturated potassium carbonate solution to each tube. The ammonia formed was then aerated and determined by titration in the usual manner.

RESULTS

The activation by cobaltous ions. Preliminary experiments confirmed the finding of Stock *et al.* (1938) that the hydrolysis of arginine by jack-bean urease in the presence of cobaltous ions is a pseudomonomolecular reaction. Two factors in the reaction were determined: first, the relationship between the concentration of cobalt ion and the concentration of

enzyme preparation, and secondly, the optimum hydrogen-ion concentration for the hydrolysis with varying concentrations of cobalt.

Fig. 1 shows that at pH 7·5 hydrolysis increased rapidly to a maximum, then fell off practically to zero as the concentration of cobalt was increased; also that there was a direct proportionality between the amount of enzyme preparation used and the amount of cobalt required to produce full activation, the total volume remaining 8 ml. These curves were lysis at pH 7·5 was obtained with 0·16mM-cobalt chloride, at pH 8·0 with 0·42mM, and at pH 9·0 with 3mM.

Activation by manganese. The optimum pH with varying metal concentrations, and the effect of incubating the enzyme and manganese for varying times before addition of the substrate were investigated. The curves obtained with increasing concentrations of manganese were of a more usual form, rising to a maximum and falling only slowly with

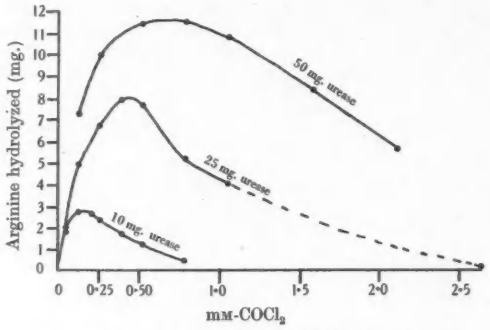

Fig. 1. Activation by cobalt at pH 7·5. Time 60 min.

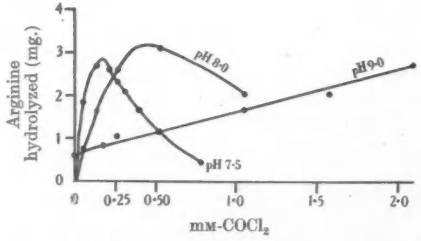

Fig. 2. Effect of hydrogen-ion concentration on cobalt activation, 10 mg. urease. Time 60 min.

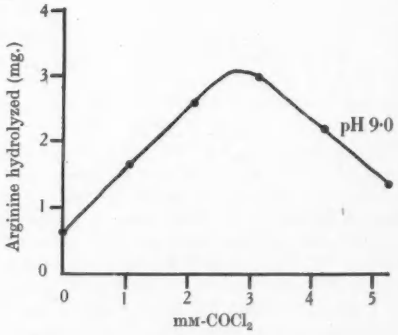

Fig. 3. Larger amounts of cobalt at pH 9·0. Time 60 min.

obtained with phosphate buffers, but a curve similar in shape was obtained with 25 mg. urease in 4 (or 5)-methylglyoxaline buffer giving a pH of 7·6 at 40°. In control experiments with urea in place of arginine, amounts of cobalt salt up to 1mM had no demonstrable effect at pH 7·5 on the activity of the urease in 10 mg. of the powder. Schmidt (1928) found that larger amounts of cobalt inactivated urease. When the hydrolysis was carried out at other hydrogen-ion concentrations, it was found that the maxima were obtained with varying amounts of cobalt, as will be seen from Figs. 2 and 3, where the maximum hydro-

further increase in concentration of manganese. The optimum pH was in the neighbourhood of 8·8, and the maximum hydrolysis was obtained with the same concentration of manganese at all reactions from pH 8·2 to 8·8 (Fig. 4). If the hydrolysis was continued for 2 hr. larger quantities of manganese produced some inhibition in a manner similar to that found with cobalt.

That the activation is not instantaneous and increases with time was shown by incubating the enzyme with manganese for varying periods up to 2 hr. before adding the substrate. A steady increase

in activation with increase in time was found under these conditions (Fig. 5).

Activation by ferrous ions. In order to obtain any activation by ferrous sulphate, relatively large quantities (3mM) had to be employed at pH 8·8 (Fig. 6), and it will also be seen that ferrous ions were inhibitory at pH 8·2. These observations, and also the reported activation of liver arginase by cysteine and ferrous salt (Purr & Weil, 1934), suggested the addition of cysteine to the reaction mixture. The addition of freshly neutralized solutions of cysteine hydrochloride to the buffered enzymes before the addition of ferrous salt activated strongly, and curves resembling in shape those seen with cobalt were obtained (Fig. 7). Control experiments without the addition of iron showed that cysteine alone in amounts greater than 2 mg. reduced the small activity of the preparation itself at pH 8·8, and that this inactivation was practically complete with 20 mg. cysteine.

The nature of the activated substance

Hellerman & Perkins (1935) found that a crude preparation of crystalline jack-bean urease could be activated to attack arginine, but later Stock *et al.* (1938) reported that the recrystallized urease is inactive. During the present investigation it was noticed that preparations of soya-bean urease were inactive toward arginine.

An attempt was made to fractionate the crude jack-bean urease. The active substance was found to be associated with the globulin fraction and was not removed by dialysis, although a very slow irreversible inactivation took place in solution even at 0°. The jack-bean globulins were therefore investigated separately. With the methods given by Sumner (1919) and Sumner & Graham (1925), crystalline preparations of 'concanavalin A' and 'concanavalin B' were prepared, which were found to be quite inactive in the presence of manganese.

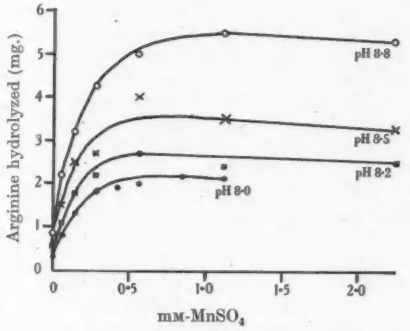

Fig. 4. Effect of hydrogen-ion concentration on activation by manganese. Time 60 min.

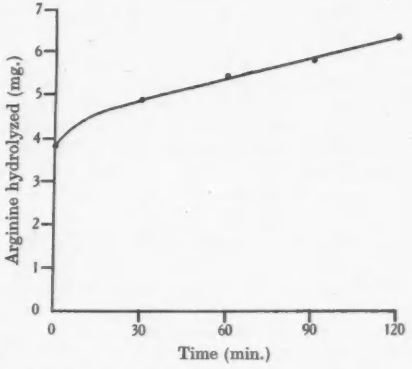

Fig. 5. Incubation of manganese and enzyme for varying times before the addition of substrate. Hydrolysis for 60 min.

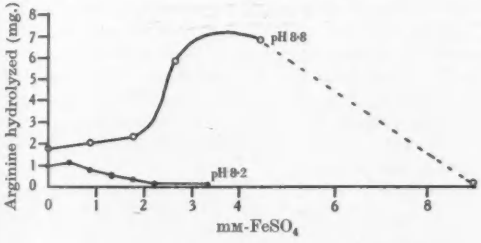

Fig. 6. Activation by ferrous iron. Time 120 min. o——o pH 8·8; •——• pH 8·2.

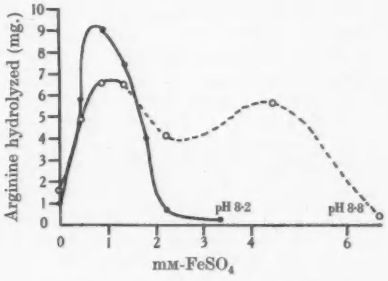

Fig. 7. Activation by ferrous iron + cysteine. Time 120 min.

The activity was found in the residual amorphous 'canavalin'. Sumner & Howell (1936) described the preparation of a fourth crystalline globulin from the amorphous 'canavalin' by the action of trypsin. A preparation of this globulin gave crystals identical in shape with those described by Sumner, but this also was found to be inactive and was not activated by manganese. In these experiments a preparation of soya-bean urease was added to those protein solutions which were devoid of urease action.

Further attempts to isolate the substance responsible have so far been unsuccessful.

DISCUSSION

The variation in the optimum pH with changes in the concentration of cobaltous ions explains the finding of Stock *et al.* (1938) of a shift in the optimal pH from 9·0 for the enzyme alone to pH 7·7 in the presence of cobalt. In their experiments the same concentration of cobalt was used throughout. The explanation of the inhibition by larger concentrations of cobalt and manganese, or by ferrous iron at pH 8·2, is not obvious. Such an inhibition by ferrous iron has already been reported for rat-liver arginase by Weil (1935). The concept of arginase as a complex of a protein carrier and a metal ion has been suggested by Edlbacher & Zeller (1936) and Edlbacher & Baur (1938). This hypothesis is supported by the work of Hellerman and his collaborators (Hellerman & Perkins, 1935; Hellerman & Stock, 1938). The finding reported here, that the activation by manganese can be increased by a preliminary incubation of the preparation with manganese, would appear to support this hypothesis. The same effect has been reported recently by Hunter & Downs (1944) for the activation of liver arginase by cobalt.

The arginase of jack-bean is very feebly active and may be compared to liver arginase after subjection to prolonged dialysis until only slightly active; such a preparation can be activated by the addition of manganese, etc. One may postulate that in the jack-bean the protein carrier is present in large excess with very little of the active metal complex.

A determination of the reduction potentials of these systems might throw light on the mechanism involved, and it is hoped to undertake work on these lines in the future. The actual component of the jack-bean extracts which is activated is still undetermined and all that can be deduced from the present experiments is that it is associated with the globulin fraction, but that it does not appear to be any of the globulins so far isolated.

SUMMARY

1. In the activation of jack-bean arginase by cobaltous ions there was a direct proportion between the amount of enzyme and the concentration of cobalt required to produce full activation. As the cobalt concentration was increased the extent of hydrolysis increased to a maximum and then fell off. The optimum pH was found to vary with the concentration of cobalt used, increasing from 7·5 to 9·0 with rising cobalt concentration.

2. With manganous ions the optimum pH was in the neighbourhood of 8·8, and was independent of the concentration of manganese. Large quantities of manganese produced some inhibition. Incubation of the enzyme with manganese before the addition of substrate caused the activity to increase with the time of incubation.

3. Ferrous salt activated only in comparatively high concentration at pH 8·8, and at pH 8·2 was inhibitory. Cysteine markedly increased the activation by ferrous ions at pH 8·8, and produced an activation at pH 8·2. The activity-concentration curves obtained with cysteine and ferrous ions were similar in shape to those obtained with cobalt.

4. The substance activated is associated with the globulin protein of the jack-bean extract, but is not concanavalin A, concanavalin B, or the fourth crystalline globulin described by Sumner & Howell (1936).

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Fumaric Acid Oxidation by Ground Preparations of Pigeon Brain

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Fumarate catalysis has been extensively studied since it was first shown that small amounts of fumaric and other C_4-dicarboxylic acids markedly increased the O_2 consumption of finely minced pigeon breast muscle (Gözsy & Szent-Györgyi, 1934; Annau, Banga, Gözsy, Huszak, Laki, Straub & Szent-Györgyi, 1935). These subsequent investigations have shown the effect to occur with many other tissues, and it is now generally believed that the catalysis is largely exerted on the pyruvate oxidation system (Annau, Banga, Blazso, Brukner, Laki, Straub & Szent-Györgyi, 1936; Annau & Erdös, 1939).

The present work has its origin in the observation of Banga, Ochoa & Peters (1939) that addition of fumarate to ground preparations of pigeon brain, respiring in a suitable medium, markedly stimulated both the O_2 uptake and pyruvate utilization. A repetition of these experiments, the details of which will be published later, has confirmed the increased O_2 uptake, but a slower rate of pyruvate disappearance has repeatedly been found. It seemed probable that this latter result might be due to the simultaneous oxidation of fumarate to pyruvate, even though this reaction does not take place in the presence of the *minced* tissue (Long, Ochoa & Peters, 1939). The work to be described shows clearly that this conversion can take place aerobically in *ground* preparations of pigeon brain, when the respiration medium contains arsenite to inhibit further oxidation of the accumulated pyruvate. Under these conditions, the activity of the fumarate oxidation system is catalyzed by inorganic phosphate and strongly inhibited by malonate, while adenine nucleotide is without effect. Observations made under anaerobic conditions, while differing in some details, have essentially confirmed these findings. A few experiments have also been carried out using *l*-malate in place of fumarate, with similar results.

EXPERIMENTAL

Materials

Fumaric and l-malic acids. The pure acids (Fraenkel), neutralized with NaOH (pH 7·3), were used. Final concentration, 0·005 M.

Oxaloacetic acid. Prepared from *dl*-malic acid (Fenton & Jones, 1900). For details of estimation, see text.

Adenine nucleotide. Three samples (Long, 1943) were used. Final concentration, 0·0003 M.

Sodium arsenite. An aqueous suspension of As_2O_3 (0·99 g.) was neutralized with 40% NaOH (pH 7·3) and diluted to 50 ml. This 0·2 M-arsenite solution was diluted as required to final concentration, 0·008 M.

Methods

(*a*) *Respiration technique.* Ground pigeon-brain preparations (suspensions), dialyzed against 0·4% KCl, were prepared as described by Long (1943); respiration was at 28° and pH 7·3 in a series of 50 ml. Erlenmeyer flasks. When O_2 uptakes were being measured, Barcroft flasks and manometers were used.

The enzyme suspension (1 ml., equivalent to 333 mg. fresh tissue) was added at zero time to the medium (1 ml.) containing fumarate, with or without arsenite, inorganic phosphate, adenine nucleotide, malonate, etc. After 40 min. respiration, the contents of the flasks were deproteinized with 25% trichloroacetic acid (1 ml.). Pyruvate was determined on measured fractions of the filtrate (Long, 1942).* Inorganic phosphate was estimated as described by Long (1943).

(*b*) *Anaerobic procedure.* The dehydrogenases of pigeon brain were prepared according to Long (1943); some indication of their relative activities can be obtained from the data in Table 1, which shows the reduction rate of methylene blue under the conditions stated.

Table 1. *Methylene-blue reduction by different substrates, using a dehydrogenase preparation from pigeon brain*

(Each Thunberg tube contained 1·0 ml. enzyme suspension (equivalent to 150 mg. fresh brain tissue), 0·2 ml. 0·5 M-phosphate buffer, pH 7·3, and 0·2 ml. 0·02% methylene blue (added from hollow stopper at zero time). Total volume 2·0 ml. Reduction at 28° *in vacuo*.)

Substrate	Reduction time t (min.)	Activity $(100/t)$
Nil	182	0·5
Fumarate	18½	5·4
l-Malate	12	8·3
Succinate	27½	3·6
α-Ketoglutarate	15	6·7
Pyruvate	16½	6·1
α-Ketobutyrate	18½	5·4

Since with fumarate or *l*-malate as H-donator, quite a visible amount of methylene blue remains in the oxidized form when equilibrium is reached, the end-point was taken

* As shown below (p. 147), the results obtained by this method include oxaloacetate as well as pyruvate. Results described as those for pyruvate in the present paper should be interpreted accordingly.

after 90% reduction, as judged by the depth of colour of a control tube containing 10% of the initial methylene blue.

The experiment of Table 1 indicates that the enzyme preparation contains appreciable amounts of fumarase and *l*-malic dehydrogenase, in addition to the α-keto-acid dehydrogenase for which the method of preparation was originally designed.

Results

The effect of fumarate and l*-malate on the* O_2 *uptake of ground and of minced preparations of pigeon brain.* Long *et al.* (1939), presenting evidence against the theory that pyruvate is oxidized by minced pigeon brain through a succinic or citric acid cycle, observed that the rate of O_2 uptake of such a tissue preparation was not increased by the addition of fumarate or *l*-malate. A repetition of this work, using ground instead of minced brain, however, has led to totally different results, the respiration in this case being markedly increased by the addition of 0·005M-fumarate (Table 2). It is probable that these results are due to the difference in permeability of the two pigeon-brain preparations towards fumarate.

Table 2. *The* O_2 *uptake of ground and of minced pigeon brain in the presence and absence of fumarate and* l*-malate*

(O_2 uptakes expressed as μl./g. fresh tissue/hr. (average of closely agreeing duplicates). 0·05M-phosphate buffer, pH 7·3, present.)

			Dialyzed brain suspension		Washed minced brain	
			O_2 uptake at 28°		O_2 uptake at 38°	
Substrate	Exp.	Dialysis (hr.)	Residual	With substrate	Residual	With substrate
Fumarate	1	3	225	387	—	—
	2	6	166	293	—	—
	3	8	117	252	—	—
	4	—	—	—	261	270
l-Malate	5	—	—	—	252	261

The conversion of fumarate to pyruvate by dialyzed suspensions of pigeon brain, respiring in the presence of varying amounts of arsenite. The increased O_2 uptake observed following the addition of fumarate to a dialyzed suspension of pigeon brain, respiring in a phosphate medium, is accompanied by the formation of only a very small amount of pyruvate. If arsenite be also present in the medium, however, the accumulation of pyruvate is greatly increased, since under these conditions its removal from the system by further oxidation is arrested. The effect of varying concentrations of arsenite on the production of pyruvate is shown in Fig. 1.

It will be observed that maximal pyruvate production is obtained in the presence of 0·002M-arsenite, higher concentrations up to 0·008M being without further effect. This observation is significant since it indicates that between these two concentrations arsenite, while inhibiting pyruvate oxidation completely, has no effect on the fumarate oxidation system, i.e. fumarase and *l*-malic dehydrogenase, unless both concentrations and those intermediate inhibit to the same extent, which is very unlikely. The literature contains two references to the effect of arsenite on the *l*-malic dehydrogenase

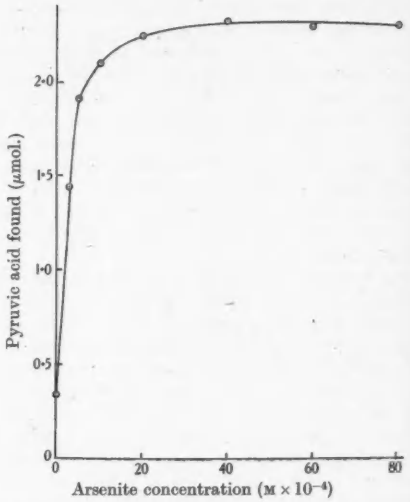

Fig. 1. The effect of arsenite concentration on the aerobic oxidation of fumarate to pyruvate. Pyruvic acid expressed as μmol./333 mg. fresh tissue/40 min.

activity of an extract from pigs' heart. Green (1936) found that 0·03M-arsenite accelerated the oxidation of *l*-malate by 38%, while Das (1937*a*) reported that 0·01M-arsenite inhibited the enzyme by 77%. These two observations are difficult to reconcile, but do not conflict with the present work since the arsenite concentrations used are somewhat higher than those employed here. Thus there seems to be no evidence that arsenite has any effect on the oxidation of fumarate under the conditions described.

The effect of inorganic phosphate on the oxidation of fumarate by ground preparations of pigeon brain. One of the most striking observations concerning the oxidation of fumarate and *l*-malate by ground preparations of pigeon brain is the dependence of the reaction rate on the concentration of inorganic phosphate in the medium. This is shown under both aerobic and anaerobic conditions.

With the Thunberg technique and methylene blue as H-acceptor, the results shown in Table 3 were obtained. It will be observed that the activity of

the enzyme system is increased 4–12 times by the presence of 0·05M-phosphate. In the absence of fumarate, phosphate does not appreciably accelerate the residual reduction rate of methylene blue.

As the phosphate concentration was varied in these experiments, the osmotic pressure and Na^+/K^+ ratio of the media were kept constant, isotonic phosphate and chloride being interchanged. The residual phosphate concentration of the tissue preparation in the total volume was equivalent to about 0·00007M.

Table 3. *The effect of inorganic phosphate on the anaerobic oxidation of fumarate and* l*-malate*

(Experimental details as in Table 1.)

		Activity (100/t)		
Substrate	Exp.	No phosphate added	0·05M-phosphate added	Ratio of activities
Fumarate	1	0·9	3·7	4·1
	2	1·6	6·3	3·9
	3	0·5	6·1	12·2
	4	0·5	4·3	8·6
l-Malate	5	1·2	5·6	4·7
	6	1·4	6·3	4·5

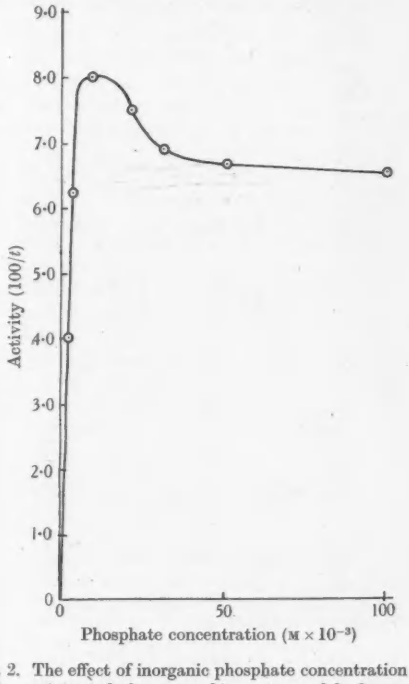

Fig. 2. The effect of inorganic phosphate concentration on the activity of the anaerobic system: dehydrogenase preparation (≡150 mg. fresh brain tissue), 0·005M-fumarate and methylene blue (0·002%).

Fig. 2 shows the effect of increasing phosphate concentration on the rate of anaerobic oxidation of fumarate. The initial portion of the curve is very steep and maximal activity of the system is reached in the presence of 0·01M-phosphate; a slight decrease in activity is observed at higher phosphate concentrations.

Under aerobic conditions, pyruvate formation from fumarate is similarly dependent on the phosphate concentration of the medium. With 0·008M-arsenite present, the conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions in the presence and absence of added 0·05M-inorganic phosphate is shown in Table 4. The residual concentration of phosphate in the tissue preparation averaged 0·001M (cf. 0·00007M for the dehydrogenase preparation).

Table 4. *The effect of inorganic phosphate on the formation of pyruvate from fumarate*

	Pyruvic acid formed (μmol./g./hr.)	
Exp.	No phosphate added	0·05M-phosphate added
1	1·9	4·9
2	2·7	8·2

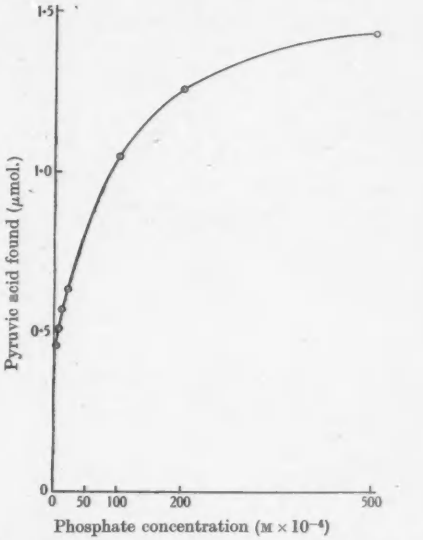

Fig. 3. The effect of inorganic phosphate concentration on the aerobic oxidation of fumarate to pyruvate. 0·008M-arsenite present.

Fig. 3 shows the pyruvate production at different phosphate concentrations. The curve differs from that obtained under anaerobic conditions, however, in that the well-defined peak at 0·01M-phosphate concentration is absent.

The effect of arsenate on the oxidation of fumarate. To a very limited extent, phosphate can be replaced by arsenate in the fumarate oxidation system. Table 5 shows the effect of increasing arsenate concentration on the conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions respiring in the presence of 0·008M-arsenite.

Table 5. *The effect of arsenate on fumarate oxidation*

(Inorganic phosphate absent.)

Arsenate concentration (M)	Pyruvic acid formed (μmol./g./hr.)
—	2·6
0·001	2·9
0·005	3·6
0·010	3·3
0·030	3·1
0·050	2·7

Maximal activity of the fumarate oxidation system is seen to be obtained when the arsenate concentration is about 0·005M, higher concentrations being less effective.

The effect of malonate on the fumarate oxidation system. Malonate is well known to inhibit both succinate oxidation and fumarate catalysis. Its effect on the oxidation of fumarate was first investigated under anaerobic conditions. Two typical experiments, in which varying malonate concentrations were used, are shown in Table 6.

Table 6. *The effect of malonate on the anaerobic oxidation of fumarate*

(Experimental conditions as in Table 1.)

Exp.	Malonate concentration (M)	Activity (100/t)
1	—	6·1
	0·0062	6·8
	0·0125	5·8
	0·0250	4·0
2	—	4·9
	0·0031	7·3
	0·0062	6·7
	0·0125	5·5
	0·0250	4·4

It will be seen that concentrations of malonate less than about 0·01M activate the anaerobic oxidation of fumarate, while higher concentrations cause inhibition. In the absence of fumarate, malonate had no effect on the activity of the residual system.

A study of the effect of malonate on the aerobic conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions in the presence of arsenite gave results of an entirely different nature. Under these conditions all concentrations of malonate inhibited the oxidation process, complete inhibition being observed in the presence of 0·03M-malonate (Table 7).

Table 7. *The effect of malonate on the aerobic oxidation of fumarate*

Malonate concentration (M)	Pyruvic acid formed (μmol./g./hr.)	Inhibition (%)
—	6·2	—
0·0005	6·1	2
0·0010	5·8	6
0·0020	5·1	17
0·0050	4·5	26
0·0100	2·8	55
0·0200	1·4	78
0·0300	0·0	100

These results may be compared with those obtained by Green (1936), who found that 0·03 and 0·3M-malonate inhibited the O_2 uptake of the *l*-malic dehydrogenase system from pigs' heart in the presence of *l*-malate, KCN, coenzyme I and methylene blue, to the extent of 11 and 47% respectively. Das (1937*b*), under comparable conditions, observed 50% inhibition of the activity of a *l*-malic dehydrogenase preparation by 0·06M-malonate. Thus it appears that at equivalent concentration, malonate is a more efficient inhibitor of the fumarate oxidation system in dialyzed pigeon-brain suspensions than of the reconstituted systems of Green and of Das.

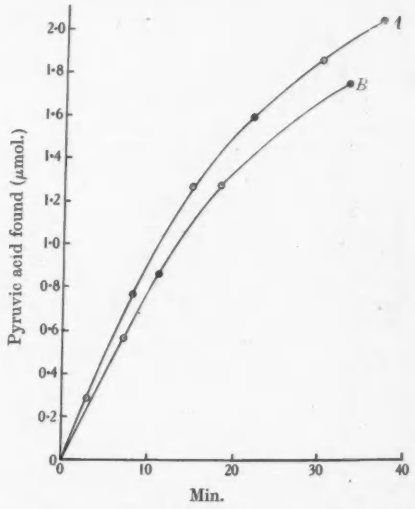

Fig. 4. The time course of the aerobic oxidation of malate (curve *A*) and fumarate (curve *B*) to pyruvate. • • adenylpyrophosphate (0·0003M) present; ⊙ ⊙ adenylpyrophosphate absent.

The oxidation of fumarate and of l*-malate in the presence and absence of adenine nucleotide.* The two curves in Fig. 4 indicate that adenine nucleotide is without effect on the oxidation of fumarate and of

l-malate to pyruvate by dialyzed suspensions of pigeon brain in the presence of 0·05M-phosphate and 0·008M-arsenite. This fact is of interest in view of the observation of Banga *et al.* (1939), that adenine nucleotide markedly increased the rate of O_2 uptake of such a tissue preparation respiring in a medium consisting of phosphate, pyruvate and fumarate. Its importance will be made clear in a later paper in which the mechanism of this latter reaction is quantitatively studied.

A comparison of the curves in Fig. 4 shows also that *l*-malate is oxidized more rapidly than fumarate in the initial stages of the reaction, and that the rates later become equal. This is interpreted as indicating that the conversion of fumarate to *l*-malate must precede oxidation, but that in the later stages of the experiment the fumarase activity is sufficiently great to make the *l*-malic dehydrogenase activity the limiting factor in the rate of oxidation.

Pyruvate or oxaloacetate as the end-product of fumarate oxidation by respiring suspensions of pigeon brain. It has been assumed in the foregoing work that the oxidation of fumarate by molecular O_2 in the presence of dialyzed pigeon-brain suspensions involves the following sequence of reactions:

(*a*) Fumarate → *l*-malate.
(*b*) *l*-Malate → oxaloacetate.
(*c*) Oxaloacetate → pyruvate.

In view of the likelihood that reaction (*c*) takes place to a limited extent only, it would be desirable to estimate the separate amounts of oxaloacetate and pyruvate produced, their sum being a function of the quantity of fumarate oxidized. This has involved an investigation of the colorimetric determination of oxaloacetate by the 2:4-dinitrophenylhydrazone method used for pyruvate (Long, 1942). As a result, it was found that oxaloacetate and pyruvate, at equivalent molecular concentration, give the same colour intensity. Thus the method, as applied to the protein-free filtrate from the respiration medium, yields the sum of the quantities of pyruvate and oxaloacetate formed, an amount which is proportional to the quantity of fumarate oxidized.

The sample of oxaloacetic acid, prepared as described under *Reagents*, contained about 20% unchanged *dl*-malic acid. This is shown by the fact that the CO_2 evolution from the β-keto-acid grouping, by aniline citrate, corresponds to only 80% of the theoretical, while the mol. wt. determination, by titration of the —COOH groups with alkali, agrees very nearly with the calculated value.

6·50 mg. required 0·891 ml. 0·1014N-NaOH. Assuming two —COOH groups, mol. wt., found = 130·7; calculated for oxaloacetic acid = 132; calculated for *dl*-malic acid = 134.

1·56 mg. in 2 ml. 0·1N-H_2SO_4, on treatment with aniline citrate, evolved 213 μl. CO_2 at N.T.P.; calculated for oxaloacetic acid = 265 μl. Purity = 80·3%.

The following experiment was carried out to estimate oxaloacetic acid by the colorimetric method of Long (1942). A series of pyruvic acid concentrations and two amounts of oxaloacetic acid were determined simultaneously. The oxaloacetic acid was corrected for its 19·7% *dl*-malic acid content, and found to give the same colour intensity as an equimolecular amount of pyruvic acid. The results are shown in Table 8.

Table 8. *The colorimetric determination of oxaloacetic acid*

	Oxaloacetic acid (μmol.)		
Drum reading	Taken	Corrected for impurity	Pyruvic acid at equivalent drum reading (μmol.)
0·443	0·382	0·306	0·310
0·566	0·509	0·408	0·400

The values given in Table 8 show that oxaloacetate is estimated as if it were pyruvate, the colorimetric method being unable to distinguish between the two. Hence the quantity hitherto estimated as pyruvate in protein-free filtrates is the sum of the amounts of pyruvate and oxaloacetate formed from fumarate, a figure which truly represents the extent of fumarate oxidation.

SUMMARY

1. The addition of small amounts of fumarate to a dialyzed pigeon-brain suspension, respiring *in vitro*, caused an increased O_2 uptake, an effect which is not observed with respiring minced brain tissue. In the presence of arsenite, pyruvic acid is formed in amounts which could be determined quantitatively.

2. The pyruvic acid formation under these conditions is catalyzed by inorganic phosphate, strongly inhibited by malonate, and unaffected by adenine nucleotide.

3. Under anaerobic conditions, with methylene blue as H-acceptor, essentially similar results have been obtained, although very low concentrations of malonate were found to be somewhat catalytic.

4. In so far as it has been studied, *l*-malate behaves like fumarate.

The author wishes to thank Prof. D. C. Harrison for his continued interest in the work.

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Properties of Azide-catalase

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It is now well established that catalase is a conjugated protein with protohaematin as its prosthetic or active group. Catalase resembles acid methaemoglobin in its colour, its absorption spectrum, and above all in its property of combining reversibly and forming well-defined derivatives with HCN, H_2S, HN_3, HF, NH_2OH, NO and $C_2H_5O.OH$. However, it differs from methaemoglobin in three important properties, namely: (1) the colour and absorption spectrum of its compound with azide (NaN_3), (2) the reaction with H_2O_2, which consists in a violent, almost explosive, decomposition of the latter, during which it is impossible to observe any changes in colour and absorption spectrum of the enzyme, and (3) the stability of its trivalent iron which is not reduced even by $Na_2S_2O_4$. In this latter respect catalase differs fundamentally from all other haematin derivatives (Keilin & Hartree, 1936).

The main object of this investigation was to examine the properties of azide-catalase in the hope of gaining further insight into the mechanism of the reaction catalyzed by free catalase.

MATERIALS AND METHODS

Catalase used for this investigation was prepared from horse liver. The initial stages of extraction are based upon those of Zeile & Hellström (1930). They have previously been described by us (Keilin & Hartree, 1936, 1938), but we have since introduced slight modifications.

5·5 kg. liver is finely minced, mixed with an equal volume of tap water and allowed to stand overnight at 10–15°. Next day, the fluid is pressed out and treated with 400 ml.* 90% ethanol/l. of fluid. The mixture is centrifuged for 30 min. and the red-brown supernatant fluid again precipitated by the addition of 200 ml.* 90% ethanol plus 100 ml. chloroform/l. When the resulting precipitate is centrifuged down a clear yellow-brown fluid is obtained with a green fluorescence. Examination of a 3 cm. layer with a small dispersion spectroscope should reveal the absorption band of catalase at 622 mμ. The fluid is left overnight at 5° when a brown precipitate containing much inactive protein is deposited. This is filtered off to give about 4 l. of a clear green-brown solution. The enzyme was removed from this solution by shaking it with three successive lots of 200 ml. calcium phosphate gel (a suspension containing 1 g. tricalcium phosphate/40 ml.), the pH being maintained at 5·6 by means of acetic acid. The combined cakes of the gel were suspended in water, brought to pH 5·6 and centrifuged. The fluid was discarded and the catalase was eluted by suspending the gel in water, adjusting the pH to 8·0 with dilute ammonia and centrifuging. Two further extractions were made with 0·02 N-ammonia. The combined eluates (790 ml.) were brought to pH 5·5 and diluted to 1 l. Addition of 250 g. $(NH_4)_2SO_4$ yielded a precipitate of ferritin plus a little catalase. This was filtered off and the bulk of the catalase precipitated by addition of a further 100 g. $(NH_4)_2SO_4$. This precipitate was filtered off, dissolved in 50 ml. water and electrodialyzed against running tap water to remove $(NH_4)_2SO_4$.

At this stage the catalase is pure enough for most purposes but can be purified further as follows: The solution is diluted to 250 ml. and treated with 50 ml. calcium phosphate gel at pH 7·0 which is centrifuged off and discarded; it is then treated with 150 ml. calcium phosphate gel in three lots at pH 6·4. The gel is centrifuged off, washed once with water and extracted five times with 0·025 M-Na_2HPO_4 (total

* The optimal volumes of ethanol should be determined by preliminary small-scale experiments.

volume of extract 450 ml.). This solution is treated with $(NH_4)_2SO_4$ (50 g./100 ml.), and the resulting precipitate is collected on a filter paper and electrodialyzed until salt-free. During the final electrodialysis the catalase often sediments to give a very concentrated solution at the bottom of the cell, depositing fine needle crystals of catalase on standing. The purity and activity of such crystals, however, do not differ from those of the mother liquor. Typical analyses of preparations obtained by this method are given in Table 1 (*a–d*).

The iron in these preparations was determined colorimetrically with $\alpha\alpha'$-dipyridyl, and the haematin was determined spectroscopically as pyridine haemochromogen (Keilin & Hartree, 1936).

The activity of catalase preparations

The activity (Kat. f.) of the enzyme, which represents the velocity constant k of the reaction divided by the dry weight of the sample used for the estimation, was determined by a method based on that of Euler & Josephson (1927) and Euler (1934). The value of the constant at zero time was determined by extrapolation from values determined at 3, 6 and 9 min. (Sumner, 1941).

Although no special attempt was made to purify our preparations by crystallization, their activities varied as a rule between 34,000 and 48,000.

These activities are higher than those obtained by Sumner & Dounce (1937) for ox-liver catalase recrystallized several times (26,000–43,000) or than those for crystalline horse-liver catalase (22,000–40,000) as given by Sumner, Dounce & Frampton (1940), who showed also by diffusion experiments that two of their preparations were homogeneous. Our preparations were less active than those of Dounce & Frampton (1939) or of Agner (1938, 1942), who give figures of 50,000–55,000 and 59,000–61,000 respectively, but attempts to confirm these values have not generally been successful (Sumner, 1941).

All liver catalase preparations contain a certain proportion of a bile pigment derivative which results from the oxidative fission of haematin groups which thereby become catalytically inactive (Lemberg, Norrie & Legge, 1939; Sumner, 1941). From the haematin content and the activity of catalase preparations one can calculate the activity of what can be described as 'complete catalase', i.e. a homogeneous protein free from bile pigment and containing four haematin groups in each molecule. On the basis of a molecular weight = 240,000 (Theorell & Agner, 1942), the haematin content would be 1·08% corresponding to an iron content of 0·093%. The figures obtained for the theoretical activity (Kat. f.) of 'complete catalase' (Table 1) are in good agreement and indicate that the total haematin in these preparations is present as the prosthetic group of the enzyme. This confirms our previous observations showing a strict parallelism between the intensities of the absorption bands and the haematin contents of various samples of catalase (Keilin & Hartree, 1936). The calculated activity of our preparations per haematin group (*d*, Table 1) is intermediate between the values of 13,000 and 15,000 obtained by Lemberg & Legge (1943) and by Sumner *et al.* (1940) respectively.

Table 1. *Haematin content and activity of different catalase preparations*

Preparation	Haematin (% of dry weight) (*a*)	Catalase activity (Kat. f.) Found (*b*)	Calc. for 'complete catalase' $(c)=\frac{b\times 1{\cdot}08}{a}$	Calc. for one haematin group $(d)=\frac{1}{4}c$
A	0·92	48,000	55,200	13,800
B	0·92	46,000	54,000	13,500
C (solution of crystals)	0·67	34,000	55,700	13,900
D (mother liquor from crystals)	0·72	36,000	54,000	13,500

General properties of azide-catalase

On addition of sodium azide to a catalase solution the colour of the solution becomes slightly more greenish, and the absorption band at 622mμ is intensified and moves about 2mμ nearer the blue end of the spectrum. Azide does not modify the general pattern of the absorption spectrum of catalase but only intensifies its main band in the red (Fig. 1). In this respect catalase differs markedly from methaemoglobin, which with azide forms a red compound with two bands at 575 and 542·5mμ (Keilin, 1936).

It can be shown spectroscopically that for the formation of azide-catalase one molecule of azide is required per molecule of haematin. The affinity of azide for catalase is, however, not as great as that of cyanide. Thus, if a solution of cyan-catalase containing two equivalents of cyanide to one of catalase haematin is treated with a standard azide solution, the absorption spectrum will indicate a 1:1 mixture of azide- and cyan-catalase only when 300 equivalents of azide are added to the solution. The affinity of cyanide for catalase is therefore 150 times greater than that of azide. Similar experiments carried out with fluoride and hydroxylamine in place of azide show that the relative affinities of these compounds for catalase at pH 7 are approximately $KCN : NaN_3 : NH_2OH : NaF = 10{,}000 : 67 : 40 : 1$. The absorption spectra of catalase and its cyanide, azide and fluoride derivatives are shown in Fig. 1.

One of the most remarkable properties which azide-catalase shares with free catalase and with its cyan-, hydroxylamine- and fluoride-derivatives is its resistance to reducing agents. Not one of these compounds can be reduced even by such a powerful reducing agent as sodium hyposulphite ($Na_2S_2O_4$). In this respect catalase differs from all known haematin derivatives.

Reaction of azide-catalase with peroxides

We have shown previously (Keilin & Hartree, 1936) that on addition of H_2O_2 or BaO_2 to a strong solution of azide-catalase the colour of the solution turns from greenish brown to red and the characteristic absorption bands are replaced by two stronger bands at 587 and 559mμ (Fig. 1, V). In about by CO we deduced that peroxides reduce the iron of azide-catalase to the ferrous compound which can be oxidized by molecular oxygen to the original ferric azide-catalase. Hydroxylamine-catalase can also be reduced by peroxides to give a spectroscopically similar autoxidizable compound.

Theorell & Agner (1942) confirmed our spectroscopic observations but offered an entirely different

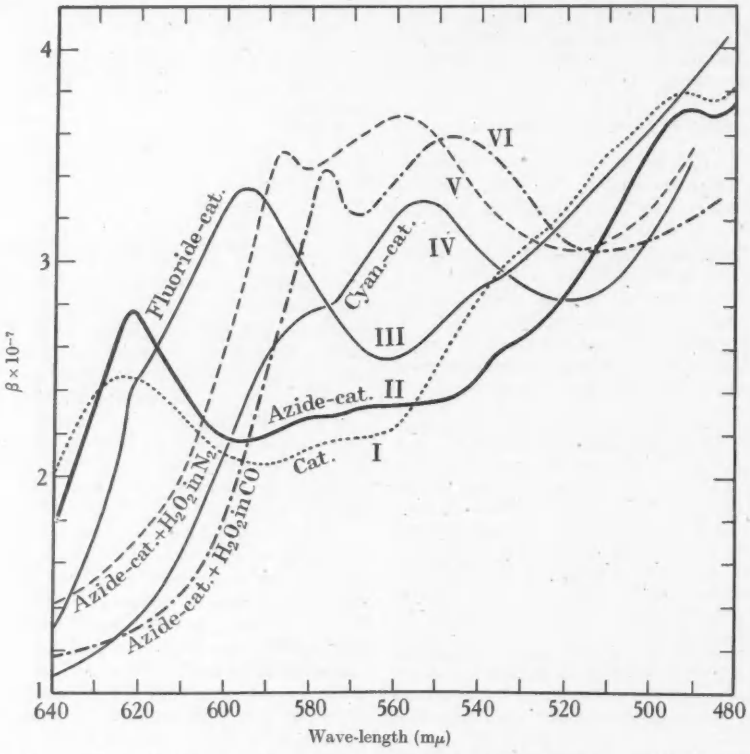

Fig. 1. Absorption spectra of catalase and its derivatives determined with the Hilger-Nutting spectrophotometer. $\beta = \frac{1}{c \times l} \log_e \frac{I_0}{I}$, where c = concentration of catalase expressed as g.-mol. haematin/ml.; l = depth of liquid layer in cm.; I_0 and I = intensities of incident and transmitted light.

Curves: I, catalase (pH 6·8); II, azide-catalase; III, fluoride-catalase; IV, cyan-catalase; V, azide-catalase + H_2O_2 in pure N_2; VI, azide-catalase + H_2O_2 in CO.

the presence of CO the two bands become more distinct and are slightly shifted towards the blue end of the spectrum. These two red compounds, especially the latter, are fairly stable in absence of oxygen, but in its presence they rapidly revert to the original azide-catalase. Even in absence of air, sufficient oxygen is liberated from the peroxide to bring about the change, though CO delays the latter considerably. From the change in spectrum brought

interpretation. The red compound obtained by treating azide-catalase with hydrogen peroxide as well as its CO derivative are considered by them to contain iron in the trivalent state. This view, they believe, is supported by the following considerations:

(*a*) 'The azide superoxide compound in N_2 shows a spectrum that so nearly resembles that of cyan-catalase that it is difficult to distinguish them' (Theorell & Agner, 1942, p. 8).

(*b*) Magnetic susceptibility values for azide-catalase treated with BaO_2 in N_2 or in CO are of the same order as those obtained for the cyanide and sulphide derivatives of catalase and are therefore incompatible with the view that these compounds contain ferrous iron.

(*c*) It is difficult to understand how azide could inhibit the oxidation of ferrohaem since 'no ferrohaemin-azide proteids are definitely known with the possible exception of verdoperoxidase' which was described by Agner (1941).

We shall now examine in detail these three considerations brought forward by Theorell & Agner.

(*a*) *Spectroscopic evidence.* We cannot agree with the statement that the absorption spectrum of azide-catalase treated with peroxide is indistinguishable from that of cyan-catalase. In fact, even when examined with a small dispersion spectroscope the absorption spectra of these two compounds appear to be different. Reference to the absolute absorption spectra (Fig. 1, IV and V) settles the point beyond dispute.

The absorption spectrum of azide-catalase treated with peroxide was determined in N_2 by using a Thunberg tube to the end of which was fused a 2 cm. cell with optical glass end plates. Hydrogen peroxide was added from the hollow stopper after filling the tube with N_2. The CO derivative was measured in the same tube in an atmosphere of CO when the shift of the bands was easily demonstrated (Fig. 1, V and VI). The spectrophotometric curves thus obtained clearly show the marked differences between the absorption spectrum of cyan-catalase and that of azide-catalase treated with peroxide in absence of oxygen.

(*b*) *Evidence from magnetic susceptibility.* In co-ordination compounds of iron the linkages may be either essentially ionic or essentially covalent independently of the valency of the iron (Pauling, 1942). With solutions containing a high concentration of haematin measurements are sufficiently precise to allow a clear indication of the valency of the iron to be obtained (Pauling & Coryell, 1936*a*), but with haemoglobin there may be deviations from the theoretical figures on account of interaction between haematin groups (Pauling & Coryell, 1936*b*). In the case of catalase and its derivatives the possibility of such interaction remains, while a further uncertainty arises from the fact that the purest liver catalase contains less than 1% haematin and the magnetic contribution of the rest of the molecule, which also contains iron in a variable amount, is difficult to assess.

The view of Theorell & Agner (1942) that azide-catalase treated with H_2O_2 in N_2 or in CO gives rise to ferric derivatives is based upon the fact that their values of the magnetic susceptibilities of these derivatives are of the same order as those of cyan- or sulphide-catalase, the ferric nature of which can be established independently. The validity of this interpretation, however, rests upon the assumption that the compound formed when azide-catalase reacts with H_2O_2 remains unchanged during the experiment. We have shown that even in purified N_2 the ferrous azide-catalase will undergo oxidation by O_2 liberated from H_2O_2 giving 25 and 50% of ferric azide-catalase within 90 and 110 min. respectively. This change is much more rapid in contact with air, and a partial oxidation can scarcely be avoided in the manipulations described by Theorell & Agner which involve transfer of the labile reduced compound from a Thunberg tube to a long open capillary suspended in a magnetic field. Under these conditions atmospheric O_2, and O_2 liberated from H_2O_2 owing to its contact with a much larger area of glass and with a celluloid septum, will accelerate the reoxidation of ferrous azide-catalase.

Theorell & Agner found the molar magnetic susceptibility of ferric azide-catalase, χ_m, to be $14{,}500 \times 10^{-6}$. They calculate the value for ferrous azide-catalase to be $\leqq 3700 \times 10^{-6}$ assuming it contains 3 ferrohaems with covalent bonds (no unpaired electrons) and 1 ferric atom attached to bile-pigment by ionic bonds (5 unpaired electrons). Their values of 6600×10^{-6} and 4920×10^{-6} for azide-catalase treated with BaO_2 in N_2 and in CO respectively can be accounted for if we assume that 25% of the reduced azide-catalase in N_2 and 19% of its CO-derivative were oxidized to the ferric state as a result of the preliminary manipulations.

Moreover, the low concentration of haematin iron in preparations of pure catalase ($< 0{\cdot}093\%$) necessitates the use of highly concentrated catalase solutions which in the experiments of Theorell & Agner contained 8·4–18·9% of pure crystalline catalase. At such concentrations it becomes difficult to suppress entirely the catalytic activity of the enzyme by azide. It is therefore difficult to avoid the formation of minute bubbles of oxygen, the paramagnetism of which may cause unpredictable changes in the values of the magnetic susceptibilities of the enzyme solutions.

We can say in conclusion that whereas the instability of the compound obtained by treating azide-catalase with H_2O_2 invalidates any determination of the valency of its iron by magnetic measurements, its reactions with O_2 and with CO clearly demonstrate that this compound contains ferrous iron.

(*c*) *Evidence based on the reaction of azide with other haematin compounds.* The fact that azide does not react with haemoglobin (Fe^{++}) although it combines with methaemoglobin (Fe^{+++}) cannot be generalized into a statement that azide reacts solely with ferric haematin derivatives and that therefore the existence of ferrous azide-catalase is improbable. In fact, although azide combines with methaemoglobin it fails to react with the ferric derivatives of haematin, parahaematin and oxidized cytochrome. It must also be remembered that the addition of azide to methaemoglobin drastically changes its

colour and absorption spectrum, yet it only slightly modifies the colour and absorption spectrum of catalase. Whereas in azide-methaemoglobin the iron atoms are bound by essentially covalent bonds (Coryell, 1939), in azide-catalase the linkages are ionic (Theorell & Agner, 1942).

The danger of generalizations of certain properties of haematin derivatives can be well illustrated by their reactions with cyanide. As is well known, cyanide forms a definite compound with methaemoglobin (Fe^{+++}) and although it does not combine with haemoglobin (Fe^{++}) it does react both with haematin (Fe^{+++}) and with haem (Fe^{++}). Moreover, within the physiological range of pH it fails to react with components *a*, *b* and *c* of cytochrome but does combine both with ferric and ferrous forms of cytochrome oxidase or the component a_3 (Keilin & Hartree, 1939).

(*d*) *Additional evidence and conclusions.* Two further indications of the reduction of azide-catalase by peroxide can be obtained by comparing the effects of different catalase inhibitors. (1) Although the affinity of cyanide for ferric catalase is about 150 times greater than that of azide, the addition of cyanide to azide-catalase previously treated with H_2O_2 in an atmosphere of pure N_2 does not cause any change in the spectrum of the ferrous azide-catalase. The α-band of this compound remains sharper than the β-band, in contrast to the cyan-catalase spectrum, in which the α-band is comparatively feeble. On replacing N_2 by CO the absorption bands shift towards the blue end of the spectrum as the CO derivative is formed. (2) The affinities of azide and hydroxylamine for ferric catalase are respectively 150 and 250 times lower than that of cyanide and yet azide and hydroxylamine are stronger inhibitors of the catalase reactions than is cyanide. This is readily explained on the assumption that cyanide reacts only with ferric catalase while azide and hydroxylamine react with both ferric and ferrous forms. The strong inhibiting action of azide and of hydroxylamine is explicable as the result of the stabilization of the ferrous state by these substances, the oxidation to ferric iron thus being greatly delayed.

The relative inhibitory effects of azide, hydroxylamine and cyanide were estimated from the concentrations of these substances necessary to inhibit by 50% the rate of decomposition of H_2O_2 by catalase.

The experiments were carried out in Barcroft differential manometers at 18° under the experimental conditions described on p. 154 except that 0·03 M-KH_2PO_4 was replaced by phosphate buffer pH 6·8 of the same concentration. A strong catalase solution was diluted and 0·2 ml. taken for each manometer giving a haematin concentration of 2×10^{-9} M. The effect of varying concentrations of the three inhibitors on the initial rate of decomposition of 0·3 ml. H_2O_2 ($\equiv 300\,\mu$l. O_2) was determined and the concentration required to give 50% inhibition was read off after plotting the results in the form: % inhibition against log concentration of inhibitor. Separate large-scale experiments showed that under these conditions $> 5\%$ of the cyanide was in the gas space as HCN. The experimental results are summarized in Table 2.

Table 2. *Inhibition of catalase activity by azide, hydroxylamine and cyanide, and the relative affinity of these substances for the enzyme*

Inhibitor	Molarity to give 50% inhibition	Relative affinity for ferric catalase (see p. 149)
NaN_3	$6{\cdot}3 \times 10^{-8}$	67
NH_2OH	$6{\cdot}3 \times 10^{-7}$	40
KCN	$4{\cdot}3 \times 10^{-6}$	10,000

From the evidence brought forward it is clear that, on treatment with H_2O_2, ferric azide-catalase undergoes reduction, thus yielding a ferrous compound. The latter shares with other ferrous haematin derivatives the properties: (1) of being stable only in absence of oxygen, (2) of undergoing a very rapid autoxidation in presence of molecular oxygen, and (3) of forming with CO a compound which is fairly resistant to oxygen. In this respect it must be remembered that there is not a single case reported of a haematin derivative combining with CO in the ferric state.

Our conclusion is strongly supported by the spectroscopic study of azide-catalase and its derivatives, and is not in the least contradicted by the measurements of magnetic susceptibilities of these compounds carried out by Theorell & Agner. Moreover, several statements in the paper by these workers actually support our view. They notice that 'the change in the position of the bands found by Keilin & Hartree when azide-catalase-superoxide is formed in CO speaks, certainly, strongly in favour of *ferro*-haemin'. They also report that 'the CO compound seems to be less sensitive to the effect of oxygen than the compound that is formed in N_2'. In carrying out measurements of the magnetic susceptibility Theorell & Agner found it necessary to protect from oxygen the azide-catalase treated with BaO_2. We fail to see why this should be necessary if the iron in the red compound thus obtained is, as they believe, in the trivalent state and there is no other part of the molecule which can undergo a reversible autoxidation.

Azide-catalase as a reagent for H_2O_2

Azide-catalase is certainly one of the most sensitive reagents for the detection of H_2O_2 formed by reduction of molecular oxygen during catalytic oxidations and autoxidations of different substances.

We have carried out these reactions in glass tubes 20 mm. high × 9 mm. diameter with fused-on optical end-plates. These tubes are placed on a microscope stage over the con-

denser and observations are carried out with the microspectroscope ocular. Sufficient catalase solution (0·2–0·5 ml.) is added to the tube to render the absorption band at 622 mμ. clearly visible. 0·1 ml. 0·1 M-phosphate buffer pH 6·8 and 5 mg. sodium azide are added when the azide compound is formed as indicated by the shift and intensification of the main absorption band. A piece of glass tubing is drawn out and bent so that it can be introduced into the tube to aerate the contents. A trace of caprylic alcohol is added to avoid frothing.

(1) *Detection of the* H_2O_2 *formed in the glucose oxidase glucose system.* If to the azide-catalase is added 0·1 ml. of a very active glucose oxidase* and 0·1 ml. 5% glucose, the colour and absorption spectrum of the solution remain unchanged. On passing a fine stream of air through this mixture its colour becomes distinctly red and spectroscopic observation shows the formation of ferrous azide-catalase indistinguishable from the compound formed on adding H_2O_2 to azide-catalase. When the air current is stopped the solution rapidly reverts to the original greenish colour of azide-catalase and shows the characteristic absorption band. If CO is present during the experiment it combines with the ferrous azide-catalase as already described. The formation of H_2O_2 during the aerobic oxidation of glucose to gluconic acid catalyzed by glucose oxidase is thus clearly demonstrated spectroscopically. In the same way the formation of H_2O_2 can be detected when the enzymes xanthine oxidase, *d*-amino-acid oxidase and uricase catalyze the aerobic oxidation of their respective substrates in presence of azide-catalase.

(2) *Detection of* H_2O_2 *during the autoxidation of sodium hyposulphite* ($Na_2S_2O_4$). The same method permits the detection of H_2O_2 during the autoxidation of $Na_2S_2O_4$. As the trivalent iron of azide-catalase, like that of catalase, cannot be reduced by this reagent, no changes are observed when azide-catalase is treated with about 5 mg. of the reagent under the conditions just described. On passing a stream of air, however, the formation of ferrous azide-catalase is at once apparent, but it persists only as long as the air stream is maintained or until the $Na_2S_2O_4$ is completely oxidized. To prevent the solution becoming too acid it is better to use disodium phosphate instead of a neutral buffer. In presence of CO the CO-derivative is rapidly formed.

Reduction of trivalent iron by H_2O_2

The reduction of the ferric iron of azide-catalase by H_2O_2 is not without parallel in the chemical behaviour of H_2O_2 which under certain conditions may act as a reducing agent. Kuhn & Wassermann (1933) showed that in presence of αα′-dipyridyl ferric salts are quantitatively reduced by H_2O_2 to the ferrous dipyridyl complex. This reaction may be demonstrated very simply by the addition of H_2O_2 to a solution of ferric chloride in presence of αα′-dipyridyl. It was therefore of interest to find whether haematin derivatives other than azide-catalase could be reduced by H_2O_2.

(1) *Reduction of alkaline methaemoglobin by* H_2O_2. Acid methaemoglobin, as we have shown previously, forms a definite compound with H_2O_2 but the iron of this derivative remains in the trivalent state (Keilin & Hartree, 1935) even in complete absence of O_2 or in presence of CO. Although it decomposes rapidly on standing, no recognizable ferrous derivatives can be detected. It is possible, however, at a higher pH to observe reduction of methaemoglobin iron.

For this purpose a catalase-free solution of methaemoglobin was prepared from recrystallized oxyhaemoglobin. It was diluted with 0·25 M-Na_2HPO_4 until it contained 14 mg. protein/ml. About 3 ml. of this solution are placed in a Thunberg tube and 0·1 ml. 20 vol. H_2O_2 placed in the hollow stopper. The tube is filled with CO by evacuation, closed and inverted. Spectroscopic examination of this solution shows that the absorption bands of alkaline methaemoglobin are gradually replaced by those of CO-haemoglobin. In other words, the trivalent iron has been reduced by H_2O_2. In a similar experiment at pH 6·8 the addition of H_2O_2 yielded a mixture of free acid methaemoglobin and its H_2O_2 compound. During 15 min. standing, complex changes took place but faint bands of CO-haemoglobin could be detected. Lemberg, Legge & Lockwood (1941) have also given evidence for the reduction of methaemoglobin by H_2O_2.

(2) *Reduction of ferric cytochrome* c *by* H_2O_2. A solution of ferric cytochrome *c* in disodium phosphate is slowly reduced to the ferrous form by very dilute H_2O_2.

This can be easily demonstrated by treating 5 ml. 0·1% oxidized cytochrome *c* in 0·2 M-Na_2HPO_4 with 0·1 ml. H_2O_2 ($\equiv 100\,\mu l.\ O_2$). Within 5 min. the sharp α-band of reduced cytochrome *c* can be seen superimposed upon the diffuse absorption band of oxidized *c*. The band eventually fades as cytochrome undergoes a destructive oxidation. This experiment may be carried out in an open tube since reduced cytochrome *c* is not autoxidizable below pH 12.

Other attempts to reduce the trivalent iron of catalase

Zeile, Fawaz & Ellis (1940) claimed that they were able to reduce H_2S-catalase by treating it with $Na_2S_2O_4$ and removing H_2S by means of a stream of N_2. The solution thus obtained was described as showing no distinct absorption bands, but when this solution was shaken with air the absorption spectrum of the original catalase returned. We attempted several times to repeat these experiments but failed to obtain the above results.

More recently Dounce & Howland (1943) claimed that from a crystalline beef-liver catalase dried in a frozen state they obtained a preparation which could be reversibly reduced with $Na_2S_2O_4$. They concluded that drying catalase in a frozen state causes 'a mild denaturation' and 'a sufficient loosening of the linkage between iron and protein to permit $Na_2S_2O_4$ to reduce the iron'. The activity of this preparation was, however, only one-third

* A sample of purified glucose oxidase (notatin) used in these experiments was kindly supplied by Prof. H. Raistrick and Dr J. H. Birkinshaw.

that of the original preparation. The similarity of the spectrophotometric curves of original and dried catalase shows only that the latter contains an undetermined amount of unmodified catalase, since absolute values for the extinction coefficient have not been used by these workers in plotting the absorption curves. The dried preparation was probably a mixture of about one-third unmodified and fully active catalase and two-thirds of a modified and completely inactive enzyme responsible for the change in absorption spectrum on reduction. The properties of such a preparation can hardly throw any light on the mechanism of decomposition of H_2O_2 by catalase.

Manometric experiments demonstrating the reduction of azide-catalase by H_2O_2

Spectroscopic experiments have clearly shown that azide-catalase is reduced by H_2O_2 to an autoxidizable ferrous compound. The inhibition of catalase activity by azide is therefore due to the stabilization of the ferrous state of the enzyme just as cyanide inhibition is due to the stabilization of the ferric state. As we have shown, the stability of ferrous azide-catalase is greater in N_2, and much greater in CO, than in air. The problem that arises is whether CO, by stabilizing the ferrous form, will inhibit the activity of catalase in presence of concentrations of azide which are in themselves too low to have a marked inhibitory effect. Although a brief answer to the question has already been given (Keilin & Hartree, 1938) we considered it important to repeat this work over a wider range of experimental conditions.

The experiments were carried out in Barcroft differential manometers at room temperature. The right flasks received 0·3–0·5 ml. of very dilute catalase, 0·3 ml. sodium azide solution of suitable concentration and 0·03 M-KH_2PO_4 to bring the volume to 3·0 ml. In a dangling tube was placed 0·3 ml. H_2O_2 (equiv. 300 μl. O_2; diluted perhydrol). By using glassware freshly cleaned with chromic acid and thoroughly rinsed, the spontaneous decomposition of H_2O_2 was found to be negligible. The left flasks received 3·3 ml. 0·03 M-KH_2PO_4. Gases were introduced into the manometers, while they were shaking, by evacuating three times to about 50 mm. Hg and allowing the gas to stream in slowly. N_2 and CO were freed from oxygen by passing them over copper heated to 400°. A mercury gasometer was used for storing the oxygen-free CO and for making up CO:N_2 mixtures. Gas stored in the gasometer sometimes picked up impurities capable of inhibiting free catalase, and it was therefore passed to the manometers via a charcoal trap. The CO:O_2 mixtures were made up over water. After introduction of the gases and equilibration of temperature the manometer taps were closed, the dangling tubes dislodged and the manometers shaken rapidly (200 strokes/min.). Readings were taken every min. for 10 min. The initial, almost constant, rate of O_2 evolution was recorded. The small concentrations of azide used exerted only a small inhibition (*c.* 25%) on the catalase.

(*a*) *The inhibition of catalase activity by* CO *in presence of varying concentrations of azide.* The right flasks of the manometers received 0·4 ml. catalase diluted 10,000 times (about $1{\cdot}9 \times 10^{-8}$ M in haematin). A pair of experiments, one in pure N_2 and one in CO, was carried out in presence of each of seven concentrations of sodium azide varying from 0 to 3×10^{-8} M. Under these conditions azide-free catalase was inhibited neither by N_2 nor by CO, when compared with air as a standard. The results of these experiments, which are summarized in Fig. 2, show that CO inhibits the activity of the

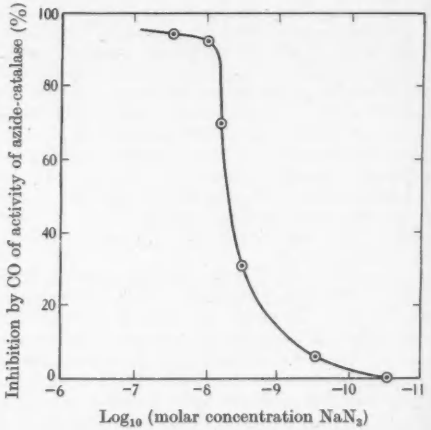

Fig. 2. Inhibitory effect of CO on the decomposition of H_2O_2 by azide-catalase. The influence is indicated of varying concentrations of sodium azide on the inhibitory action of CO compared with pure N_2 as standard. Catalase haematin, $1{\cdot}86 \times 10^{-8}$ M.

enzyme by more than 90% at azide concentration reaching 10^{-8} M, which corresponds to about half the calculated concentration of catalase haematin present at the beginning of the experiment. The fragility of the very dilute catalase in the flask, which increases markedly in presence of CO, may certainly account for a destruction of 50% of the catalase during the preliminary manipulations. In this case the position of the inflexion of the curve indicates the presence of azide and catalase components in a 1:1 ratio.

(*b*) *The activity of azide-catalase in* CO:O_2 *and* CO:N_2 *mixtures.* In these experiments a more concentrated solution of catalase was used (approximately 4×10^{-7} M in haematin) together with 10^{-6} M-sodium azide. The percentage inhibition of enzyme activity in CO:N_2 mixtures was estimated by comparison with the activity of a control experiment in

N_2,* whereas the activity in $CO:O_2$ mixtures was compared with that in the corresponding $N_2:O_2$ mixtures. The enzyme activity in all control experiments showed no appreciable variations. The rate of decomposition of H_2O_2 was measured over a period of 1 min. because with a shorter period consistent results could not be obtained, while longer periods would introduce progressively larger changes in the composition of the gas phase. The experimental results are summarized in Fig. 3 (I, II). The

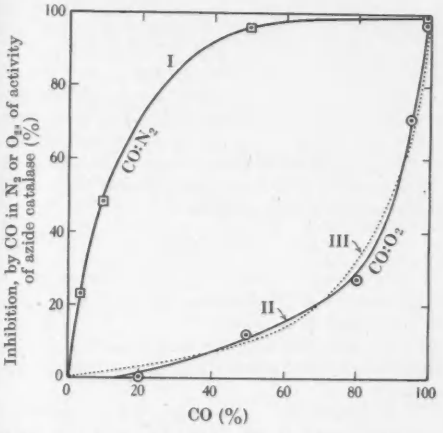

Fig. 3. Inhibitory effect of CO on the decomposition of H_2O_2 by azide-catalase. 4×10^{-7}M-catalase haematin and 10^{-6}M-sodium azide. Effect of: (I) $CO:N_2$ compared with N_2; (II) $CO:O_2$ compared with $N_2:O_2$; (III) theoretical curve for $CO:O_2$ mixtures, assuming the partition constant $k=9$.

curve for $CO:N_2$ mixtures is in fact a curve for these mixtures plus the quantity of O_2 produced during 1 min. Curve I will therefore occupy a position intermediate between curve II and a theoretical curve of inhibitions at zero time. The deviation of I from such a theoretical curve will increase with the rate of liberation of oxygen and therefore will be greatest when the percentage inhibition is smallest (i.e. in small partial pressure of CO). We may therefore postulate that the theoretical $CO:N_2$ curve will correspond to a virtually complete inhibition of the reaction for all $CO:N_2$ ratios, whereas the position of I is variable, depending upon the time interval over which the decomposition of H_2O_2 is measured. Curve II for $CO:O_2$ mixtures, on the other hand, may be considered as significant, as the rate of O_2 production will not appreciably alter the $CO:O_2$ ratio except at vanishingly small O_2 pressures. In $CO:O_2$ mixtures there is a definite competition between the two gases for the ferrous azide-catalase, the enzyme having a greater affinity for O_2 than for CO. The partition constant of azide-catalase between O_2 and CO can be expressed as

$$k = \frac{[\text{Cat. } O_2]\,[CO]}{[\text{Cat. } CO]\,[O_2]},$$

where Cat. O_2 and Cat. CO denote the concentration of active and inactivated enzyme respectively. The value of k for the preparation examined is approximately 9 (III, Fig. 3) and hence of the same order as k for cytochrome oxidase (Keilin, 1930). However, as this result was based only upon two sets of experiments the possibility of a variation in the value of k according to experimental conditions cannot be overlooked. It is important to note that the portion of the enzyme inhibited by CO may comprise two fractions: one reversibly and the other irreversibly inactivated. The irreversible inhibition may be due to the fact that the stabilizing of the azide-catalase in the ferrous state makes its porphyrin rings more fragile and so more liable to undergo oxidative fission by H_2O_2 (Lemberg *et al.* 1941). However, we have been able to confirm our previous experiments showing that the inhibition of azide-catalase by CO is to a great extent restored by the radiation from a mercury vapour lamp. Thus the irreversible inhibition by CO can occur to a small extent only.

We have carried out a large number of manometric experiments in phosphate buffer, pH 6·8, with results similar in every way to those already described. We have also obtained analogous results using hydroxylamine in place of azide.

DISCUSSION

Although catalase can be easily purified and obtained in a concentration suitable for direct spectroscopic examination, the great difficulty in the study of the mechanism of its reaction with H_2O_2 is the violent decomposition of the latter unaccompanied by any visible change in the colour or absorption spectrum of the enzyme.

If an intermediate compound is formed in this reaction, it must have much too short a life to be detected spectroscopically. It is only by considerably slowing down the velocity of the reaction that

* Under these conditions the catalytic activities in air and N_2 are identical, although the spectroscopic experiments lead us to expect an inhibition in N_2. However, the ratio (catalase)/(H_2O_2) is 20,000–50,000 times higher in the spectroscopic than in the manometric experiments. One can therefore expect differences of a similar order of magnitude in the ratio (rate of O_2 production)/(catalase iron), and when this ratio is high the chances of stabilizing an autoxidizable ferrous form in N_2 become remote. By using 0·5 ml. undiluted catalase and 0·3 M-azide in Barcroft manometers the conditions are closer to those of the spectroscopic experiments and, under these circumstances, about 50% inhibition in N_2 can be obtained.

one could hope to detect such an intermediate compound. This can be achieved by studying the reaction in the presence of certain inhibitors. It has been found that whereas the inhibition of the catalase reaction by cyanide and sulphide is due to the stabilization of the trivalent iron of catalase, thus preventing the formation of an intermediate compound, the inhibition by azide or by hydroxylamine is due to the stabilization of an intermediate ferrous compound of catalase, thus inhibiting its reoxidation to the original ferric state. The compound which is obtained by treating azide-catalase with H_2O_2 shares with ferrous haematin derivatives the property of being autoxidizable and of combining with CO.

Considering that the combination of catalase with azide is perfectly reversible and that azide-catalase has many properties in common with free catalase (such as colour, absorption spectrum, magnetic susceptibility and resistance to reducing agents), it would appear that the reaction of azide-catalase with H_2O_2 is the best model reaction for the study of the mechanism of the catalytic decomposition of H_2O_2 by free catalase.

The fact that ferric azide-catalase can be reduced by peroxide to a ferrous compound which can be reoxidized by molecular oxygen suggested that the catalytic activity of the free catalase is based upon similar reactions. The two equations which we proposed in a previous paper (Keilin & Hartree, 1938):

$$4Fe^{+++} + 2H_2O_2 = 4Fe^{++} + 4H^+ + 2O_2, \quad (1)$$

$$\frac{4Fe^{++} + O_2 + 4H^+ = 4Fe^{+++} + 2H_2O}{2H_2O_2 = 2H_2O + O_2} \quad (2)$$

are nothing more than a general outline of the over-all reaction experimentally demonstrated for azide-catalase, involving (1) changes of valency of catalase iron and (2) oxidation of the ferrous catalase by molecular oxygen. Any other equations adequately expressing these two facts would be equally acceptable.

The view that the catalytic activity of free catalase is accompanied by changes in the valency of its iron has already been suggested by other workers (Haber & Willstätter, 1931; Weiss, 1937).

However, the failure to demonstrate manometrically for the free catalase the importance of oxygen in this reaction is not surprising if the affinity of ferrous catalase for oxygen is very great.* It is conceivable that even in an atmosphere of nitrogen sufficient oxygen will be available either from the spontaneous decomposition of the H_2O_2 or from the initial reduction of ferric iron (reaction 1) to initiate the catalatic reaction. The induction period of this reaction with its autocatalytic cycle may well be completed within the first few seconds before it can be registered experimentally in a manometer.

Nevertheless, the study of azide-catalase strongly supports the view that the activity of free catalase involves changes in the valency of its haematin iron and that the ferrous catalase is probably reoxidized by molecular oxygen. So far azide-catalase has provided us with the best, if not the only, available line of approach to the experimental study of the mechanism of the catalase reaction.

If further evidence is called for it should be based upon the results of experiments with the enzyme rather than upon purely theoretical discussion of plausible reactions derived from the study of the activity of inorganic catalysts showing only superficial resemblance to catalase.

* Our previous experiments, in which we believed to have demonstrated manometrically the part played by oxygen in this reaction, could not be confirmed. As we have recently shown (Keilin & Hartree, 1943), the depression of the catalytic activity of the free enzyme was due in these cases not to the absence of oxygen but to the presence of minute traces of oxides of nitrogen formed by passing the nitrogen over hot copper at a temperature well above 400°.

SUMMARY

1. Catalase combines reversibly with azide. As in the case of free catalase, the haematin iron of azide-catalase is trivalent.

2. The formation of this compound requires one molecule of azide per iron atom of catalase haematin.

3. Azide-catalase resembles free catalase in colour, absorption spectrum, magnetic susceptibility and resistance to reducing agents such as $Na_2S_2O_4$.

4. On addition of H_2O_2 to azide-catalase, the colour of the compound turns from greenish brown to red and its absorption band in the red is replaced by two bands at 587 and 559 mμ.

5. That the compound thus obtained contains iron in the divalent state is supported by the following evidence:

(*a*) The compound is stable only in absence of O_2; in its presence it rapidly reoxidizes to the original azide-catalase.

(*b*) Like other ferrous haematin derivatives it reacts with CO, forming a well-defined compound which is more stable in air.

(*c*) The decomposition of H_2O_2 by azide-catalase is inhibited by CO and this inhibition is abolished by light.

(*d*) Cyanide has a much greater affinity for catalase than has azide and can therefore displace it from ferric azide-catalase. After treatment of azide-catalase with H_2O_2, however, cyanide produces no such effect.

(*e*) Contrary to the view expressed by Theorell & Agner (1942), the absorption spectrum of reduced azide-catalase is quite different from that of ferric cyan-catalase.

(*f*) Measurements of the magnetic susceptibility of the compound in N_3 and in CO carried out by Theorell & Agner do not in any way contradict the view that the iron of this compound is in the ferrous state.

6. The close relationship between the properties of azide-catalase and the free enzyme suggests that the catalytic decomposition of H_2O_2 by catalase involves a cyclical reduction and oxidation of its iron, as has been established in the case of azide-catalase.

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The Chemical Conversion of Di-iodotyrosine into Thyroxine

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The fact observed by Ludwig & von Mutzenbecher (1939), that iodination of certain proteins under controlled conditions gave rise to products from which thyroxine could be isolated, could be explained in one of two ways: either the proteins in question contained thyronine as a hitherto unrecognized constituent which was directly converted by iodination into thyroxine, or the tyrosine of the protein was iodinated to 3:5-di-iodotyrosine, part of which subsequently formed thyroxine. The latter mode of formation of thyroxine had indeed been suggested by one of us many years ago (Harington & Barger, 1927) and evidence, both chemical and biological, which had accumulated over a number of years (cf. Harington, 1944) favoured the supposition; its likelihood was further increased by the later observation of von Mutzenbecher (1939) that di-iodotyrosine on incubation in alkaline solution spontaneously gave rise to traces of thyroxine.

The over-all reaction representing the conversion of di-iodotyrosine into thyroxine can be crudely formulated as involving the loss, between two molecules of di-iodotyrosine, of one molecule of alanine; in such a formulation no oxidative process need necessarily be assumed. In actual fact, however, it is scarcely possible to conceive such an intermolecular dissociation, and it seems almost certain that the first stage of the reaction must be an oxidative process whereby the molecule of di-iodotyrosine is converted into one or more less stable and more reactive forms.

The supposition that oxidation must play a part is strengthened by a further consideration. By altering the conditions of iodination of a protein two entirely different results can be obtained. If the experiment is conducted as rapidly as possible in cold concentrated solution in presence of excess of ammonia the predominant and sometimes the

exclusive result is substitution of the tyrosine groups to form 3:5-di-iodotyrosine (cf. Neuberger, 1934; Harington & Neuberger, 1936). If, on the other hand, it is desired to form products containing thyroxine similar to those prepared by Ludwig & von Mutzenbecher the iodination must be conducted in warm dilute solution at a slightly alkaline reaction (pH about 8), and the thyroxine content of the products is increased by prolonged incubation of the reaction mixtures under aerobic conditions. In the present paper direct evidence will be given that oxidation is involved in the conversion of di-iodotyrosine into thyroxine, since this conversion fails when the incubation of di-iodotyrosine in alkaline solution is conducted in the complete absence of oxygen.

The suggestion that the formation of thyroxine during iodination of proteins under these conditions and during incubation of solutions of di-iodotyrosine was essentially an oxidative process was developed by Johnson & Tewkesbury (1942) who postulated hypoiodite as the effective oxidizing agent in both cases; these authors indeed claimed increased yields of thyroxine as the result of addition of hypoiodite to an alkaline solution of di-iodotyrosine, although they did not offer experimental evidence. Further reference to the question of the actual oxidizing agent involved will be made below.

Johnson & Tewkesbury proposed in outline a theory of the process involved in the formation of thyroxine from di-iodotyrosine and this was further developed by one of us (Harington, 1944) on the basis of a general consideration of the conditions of the reaction and of additional results obtained by ourselves. The present paper contains an account of these results and of further work which strengthens the basis upon which the theoretical treatment referred to was founded.

Formation of thyroxine by incubation of di-iodotyrosine

The theory of the oxidative conversion of di-iodotyrosine into thyroxine to which reference has been made was developed on the assumption that the process involved the amino-acid in the form of the phenoxide ion; such an assumption seemed justified by the fact that both under the conditions employed by von Mutzenbecher and under those obtaining *in vivo*, where the same transformation presumably occurs, the phenolic group of di-iodotyrosine will be largely ionized. (According to Winnek & Schmidt (1935) this group has pK 6·5.) The first matter to be considered therefore is the effect of pH on the formation of thyroxine under von Mutzenbecher's conditions, i.e. on incubation of di-iodotyrosine at 38° for 14 days. The results of our experiments on this point are summarized in Table 1. From this it appears that there is no formation of thyroxine in acid solution, whilst on the alkaline side the formation attains a maximum at about pH 10 and falls off sharply at higher alkalinities. In considering these results it must be borne in mind that the pH values recorded are those at the beginning of the incubation; during the reaction acidic products are formed which cause a gradual fall of the pH.

Table 1. *Formation of thyroxine by incubation of di-iodotyrosine for* 14 *days at* 38°

Initial pH	Destruction of di-iodotyrosine (%)	Net yield of thyroxine (%)
1·0	22	0
7·8	36	0·74
8·8	22	1·53
10	22	2·82
13	17	0
14	13	0

Clearly the fact that formation of thyroxine becomes significant at pH 7·8, which results from addition of 1 molecular equivalent of sodium hydroxide to di-iodotyrosine, and reaches its maximum at pH 10, that is to say after 1·95 equivalents of alkali have been added, is in accordance with the idea that it is the phenoxide ion which is being oxidized. Discussion of the reason for the absence of thyroxine formation at higher alkalinities is best postponed until further experiments have been described.

Effect of added oxidizing agent on the formation of thyroxine

If the formation of thyroxine from di-iodotyrosine is in fact an oxidative process it should be possible to accelerate it and to obtain greater yields by addition of an appropriate oxidizing agent. It was pointed out by Johnson & Tewkesbury that the process seems to bear close resemblance to certain reactions observed by Pummerer, Melamed & Puttfarcken (1922) and Pummerer, Puttfarcken & Schopflocher (1925) in the course of their study of oxidation of phenols by alkaline ferricyanide; we therefore first investigated the action of potassium ferricyanide on di-iodotyrosine under the conditions employed by Pummerer *et al.* for the oxidation of simple phenols such as *p*-cresol; in no experiment of this type, however, were we successful in isolating thyroxine, or indeed any other pure oxidation product. Under the mildest conditions which we could devise, ferricyanide caused extensive disruption of the di-iodotyrosine with the production of intractable tars. A similar experience has recently been reported by Barkdoll & Ross (1944).

We therefore considered alternative oxidizing agents and selected hydrogen peroxide, largely

because the action of this so often simulates the over-all effects of biological oxidations.

In Table 2 is recorded a series of experiments which show that hydrogen peroxide is indeed effective in accelerating the formation of thyroxine from di-iodotyrosine at 38°, although the ultimate yields are somewhat lower. Comparison of Tables 1 and 2 shows that the figures for the destruction of di-iodotyrosine and the yield of thyroxine are of the same order after 15 hr. in presence of hydrogen peroxide as they are after 14 days of simple incubation; moreover, the effects of pH on thyroxine formation are similar in both cases, the maximum at pH 10 being clearly defined both with and without peroxide; an exception to this statement is provided by the low yield obtained at pH 8·8 in presence of peroxide; this may be related to intrinsic instability of thyroxine (see below).

Table 2. *Oxidation of di-iodotyrosine by hydrogen peroxide* (0·5 *atom. equiv.* O) *for* 15 *hr. at* 38°

Initial pH	Destruction of di-iodotyrosine (%)	Net yield of thyroxine (%)
1·0	18	Trace
7·8	26	0·75
8·8	21	0·36
10	17	1·42
13	15	0·24
14	13	0

Effects of elevation of temperature and of increasing amounts of hydrogen peroxide on yield of thyroxine

The favourable effect of hydrogen peroxide on the formation of thyroxine from di-iodotyrosine as indicated by the acceleration of the process led to further experiments under more drastic conditions. In Table 3A are shown the results of oxidations of di-iodotyrosine with hydrogen peroxide at varying pH values and at elevated temperature (90–100°); under these conditions the reaction was, as might be anticipated, still further accelerated, being complete within an hour; the destruction of di-iodotyrosine was much more extensive than at the lower temperature whilst the maximum yield of thyroxine was approximately the same. Moreover, the effect of pH on the reaction remained unaltered.

At this point it became clear to us that we were unlikely to make much further progress in increasing the yield of thyroxine without introducing a new condition, since it was apparent that such thyroxine as might be formed would itself be subject to the destructive effect of hydrogen peroxide which is so obvious in the case of di-iodotyrosine. We therefore decided to attempt to make use of the observation of Leland & Foster (1932) that from an alkaline solution containing both di-iodotyrosine and thyroxine the latter is preferentially extracted by butanol. Accordingly we made further experiments in which the solution of di-iodotyrosine undergoing oxidation with hydrogen peroxide was shaken with butanol, the butanol layer being separated and renewed from time to time during the reaction. As is shown by the figures in Table 3B this method met with some success, the maximum yield of thyroxine obtained being higher than that recorded in any previous experiment.

Table 3. *Oxidation of di-iodotyrosine with excess hydrogen peroxide* (5 *atom. equiv.* O) *at* 90–100°

	A. Without butanol		B. With butanol	
Initial pH	Destruction of di-iodotyrosine (%)	Net yield of thyroxine (%)	Destruction of di-iodotyrosine (%)	Net yield of thyroxine (%)
1·0	34	Trace	—	—
7·8	100	Trace	41	0·23
8·8	90	0·13	—	—
10	46	1·63	26	3·19
13	20	0	18	2·13
14	—	—	16	0

In all the experiments recorded in Table 3 the amount of peroxide employed was greatly in excess of the theoretical, corresponding in fact to 5 atomic equivalents of oxygen. This particular proportion of peroxide had been adopted empirically as the result of a number of orientating experiments, and it became desirable to find whether the yield of thyroxine could be improved by using the butanol technique in conjunction with smaller or greater amounts of oxidizing agent at the optimum pH of 10. Table 4 shows that the yield of thyroxine is surprisingly little affected by increasing the amount of peroxide; this result is probably to be explained by formation of products in the course of reaction which catalyze the decomposition of hydrogen peroxide, so that successive additions of the latter become progressively less effective; such an effect is indeed visually obvious.

Table 4. *Effect of increasing amounts of hydrogen peroxide on formation of thyroxine from di-iodotyrosine at* 90–100° *at* pH 10 (*butanol technique*)

H_2O_2 added (atom. equiv. of O)	Destruction of di-iodotyrosine (%)	Net yield of thyroxine (%)
1·7	27	2·8
5	30	3·2
15	39	3·4
25	42	4·0

Effect of pH

It is now necessary to give some further consideration to the effect of pH on the formation of thyroxine from di-iodotyrosine. It will have been noticed that this effect is the same under all the conditions which have been studied and that, in so far as it involves absence of formation of thyroxine in the acid range and maximum formation at the point where disso-

ciation of the acidic groups of di-iodotyrosine becomes complete, it is in accordance with expectation on theoretical grounds. The outstanding question is the reason for the failure of thyroxine formation at higher alkalinities.

The first point to be noted in this connexion is the peculiar behaviour of di-iodotyrosine itself, which is illustrated by the curve in Fig. 1.* This

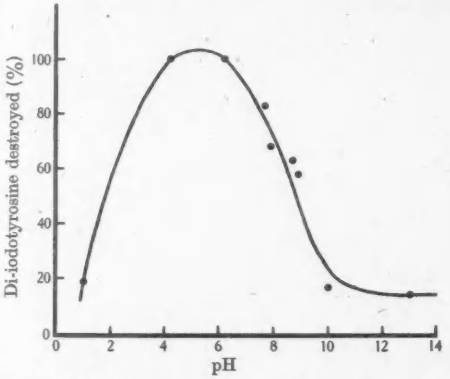

Fig. 1. Effect of pH on destruction of di-iodotyrosine by heating in solution

curve shows the relative proportions of di-iodotyrosine which are destroyed (with the formation of amorphous products) by heating for 1 hr. at 100° in aqueous solution at different pH values; a range of instability of the amino-acid is revealed which we are unable to explain, although it is perhaps noteworthy that this instability is low at pH values where the ionizable groups of di-iodotyrosine are completely dissociated; whatever the explanation this property of di-iodotyrosine must evidently influence the interpretation of our results. It is clear that under the conditions of our experiments with hydrogen peroxide two processes at least must be going on, namely, the destruction of di-iodotyrosine due to its intrinsic instability and the specific oxidation to thyroxine, and that the final result observed in the form of the yield of thyroxine represents the summation of these two and probably other reactions. It will be noticed that in the region of high alkalinity, where formation of thyroxine fails completely, di-iodotyrosine is extremely stable; at pH 10, where oxidative formation of thyroxine is maximal, di-iodotyrosine is also stable, but, as already pointed out, acid production during the course of oxidation will cause a fall of pH during the reaction and the region of instability will be entered. It is also, perhaps, of significance that di-iodotyrosine is in its region of greatest instability at the reaction of the body tissues. It is unfortunate that no conclusive information can be obtained concerning the formation of thyroxine at reactions in the neighbourhood of the isoelectric point of di-iodotyrosine (pH 4·3: Winnek & Schmidt, 1935), but the insolubility of the amino-acid and the inapplicability of the butanol technique make experiments at this pH impracticable.

A further question which must be considered in relation to the pH effect is the nature of the oxidizing agent actually responsible for the conversion of di-iodotyrosine into thyroxine. As we have seen, the optimum reaction for this conversion is pH 10. When di-iodotyrosine is incubated at this reaction iodine is slowly liberated and can be detected in solution; it will naturally be converted in part into hypoiodite. The action of hydrogen peroxide on di-iodotyrosine also brings about liberation of iodine, and the possibility exists that it is this effect rather than its direct oxidizing action on intact di-iodotyrosine which is responsible for the favourable effect of peroxide on formation of thyroxine.

Whilst there seems to be no certain means of determining whether hydrogen peroxide in our experiments is effecting the conversion of di-iodotyrosine into thyroxine by direct oxidation or by the liberation of iodine, which then brings about the reaction, it appears to us that there are two cogent reasons in favour of the latter supposition; these reasons are the significant degree to which the reaction proceeds in absence of peroxide, and the effect of pH. If peroxide were itself the effective oxidizing agent it would be natural to expect a greater increase than we have been able to observe in the yields of thyroxine obtainable in its presence, as compared with those resulting from simple incubation of di-iodotyrosine under aerobic conditions; moreover, it is not obvious why the effect of peroxide, if this were indeed to produce thyroxine directly, should fall off so sharply between pH 10 and 13.

Accepting the probability that in presence as in absence of peroxide the liberation of iodine is the decisive event, we may consider what this implies as to the nature of the actual oxidizing agent concerned. The two oxidants to be considered are iodine and hypoiodite. It was stated by von Mutzenbecher (1939) (without experimental details) that the formation of thyroxine on incubating di-iodotyrosine in alkaline solution was unaffected by the presence of thiosulphate, although it was prevented by sulphite. If this statement were correct it would exclude both iodine and hypoiodite as the effective agent since both are reduced by thiosulphate; in fact, however, we have ourselves found that the formation of thyroxine is almost completely inhi-

* Since this was written a paper by Kraft & Denzel (*Hoppe-Seyl. Z.* 1942, **272**, 147) has come to our notice where similar observations have been made in the alkaline range.

bited by the presence of thiosulphate, so that as far as this experiment is concerned both iodine and hypoiodite remain possibilities. It has already been mentioned that Johnson & Tewkesbury (1942) claimed support for their theory, that hypoiodite was the effective agent, by the increased yields of thyroxine obtainable after addition of hypoiodite to the reaction mixture. Even if this claim had been substantiated, however, it would not constitute conclusive evidence; the equilibrium between iodine and hypoiodite is governed by pH and thus, unless the pH were simultaneously raised, addition of 'hypoiodite' would in fact mean the increase in concentration both of hypoiodite itself and of free iodine. Assuming the correctness of the claim of Johnson & Tewkesbury therefore, their result could be explained equally well by the supposition that it was the increase in the concentration of free iodine which was effective. We actually describe below an experiment in which iodine is added to a solution of di-iodotyrosine at pH 10 with resulting formation of thyroxine in significant amount; this is, however, no more conclusive than the experiment of Johnson & Tewkesbury since we are merely approaching the same equilibrium mixture of iodine and hypoiodite from the other side.

We ourselves indeed incline strongly to the view that it is iodine and not hypoiodite which effects the oxidation of di-iodotyrosine to thyroxine. If hypoiodite were responsible there is no apparent reason why the reaction should fail above pH 10, whilst if free iodine were needed such a failure would be expected; it is indeed just in the pH range where iodine is completely converted into hypoiodite that failure of the oxidation occurs. The supposition that free iodine is the effective oxidizing agent also offers a reasonable conception of the events in the biological formation of thyroxine; there is now ample evidence that this formation occurs from di-iodotyrosine and the experiment of Keston (1944) makes it likely that iodine participates in the reaction. At the pH of the body fluids hypoiodite can exist only in minute concentration; on the other hand, in presence of an enzymic oxidizing system capable of liberating iodine from iodide, all the conditions which we have supposed to be necessary for thyroxine formation would be operative; the free iodine produced would have the dual function of reacting with tyrosine to produce di-iodotyrosine and of oxidizing the latter to thyroxine. The conditions existing in the thyroid gland on this supposition would indeed be closely similar to those employed by Ludwig & von Mutzenbecher (1939) in their experiments on the iodination of proteins.

DISCUSSION

It appears to us that our results leave no doubt of the oxidative nature of the conversion of di-iodotyrosine into thyroxine. Proof of this rests on the failure of the conversion in absence of oxygen and on the significant increase of yield and acceleration of the reaction which can be effected by the addition of an oxidizing agent.

The system as we have studied it is, however, greatly complicated by the peculiar intrinsic instability of di-iodotyrosine in the range of pH with which we are concerned, and by the fact that changes in pH not only affect the degree of ionization of di-iodotyrosine but may also influence the concentration of the effective oxidizing agent. It must further be reiterated that in speaking of pH we refer only to the initial value; a fall of 0·5–1 pH unit may occur during the oxidation. In these circumstances an adequate interpretation of the reaction cannot be given but, taking all the observed facts into account, we would offer the following tentative conclusions.

The fact that formation of thyroxine is not observed at pH values below that at which the phenolic group of di-iodotyrosine is significantly dissociated supports the hypothesis that the oxidation involves the phenoxide ion. If this assumption is accepted the failure of thyroxine formation at high alkalinity is to be explained (*a*) by increased stability of di-iodotyrosine and (*b*) by ineffectiveness or removal of the oxidizing agent. Reality is given to the possibility (*b*) by the supposition that the effective oxidizing agent is iodine, which is known to be liberated by simple aerobic oxidation of di-iodotyrosine and more rapidly by oxidation of the latter with hydrogen peroxide. The increasing yields of thyroxine over the range pH 7·8–10 would thus be explained by the favourable effect of increasing ionization of the acidic groups of di-iodotyrosine while free iodine is still present, whilst the failure of thyroxine formation at pH > 13 would be due to the absence of free iodine from the system.

Admittedly this interpretation leaves much to be desired in precision and completeness. Other unknown factors may come into play such as an intrinsic instability of thyroxine, similar to that observed for di-iodotyrosine but operative over a different range of pH. The interpretation given is, however, in accordance with the facts as we have observed them, and it is perhaps worthy of consideration in its biological implications.

EXPERIMENTAL

Formation of thyroxine by incubation of di-iodotyrosine at 38°

The following experiment is typical of the series, the results of which are shown in Table 1. The example chosen for detailed description is that which imitates as closely as possible the conditions described by von Mutzenbecher (1939).

l(+)-Di-iodotyrosine dihydrate (11·75 g.) was dissolved in 0·1 N-NaOH (25 ml.); the solution was adjusted to pH 8·7 by addition of N-NaOH (3 ml.) and incubated at 38° for 14 days. At the end of this time 12·5 N-NaOH (20 ml.) was added and the mixture was twice extracted with its own volume of *n*-butanol; the combined extracts were washed once with an equal volume of 2 N-NaOH and evaporated under diminished pressure. The residue was taken up in water (50–60 ml.) and the solution was acidified at the boiling point.

The precipitate was collected from the cooled solution and dried in a desiccator (152 mg.); it was then extracted with ether and the ether-insoluble portion (78 mg.) was dissolved in the minimum of boiling 0·1 N-K_2CO_3 (about 5 ml.); the filtered solution was kept overnight in the ice-chest. The white and crystalline potassium salt which had separated was converted into free crystalline thyroxine by solution in aqueous-ethanolic alkali and acidification at the boiling point with acetic acid. Yield, 32 mg.

The original aqueous solution was freed from butanol by evaporation under diminished pressure, and strongly acidified at the boiling point; a tarry precipitate was removed by rapid filtration of the hot solution and the pH was adjusted to about 5. The di-iodotyrosine which separated on cooling, together with a second crop obtained by concentrating the mother liquor, amounted to 9·22 g. or 79·5% of the starting material.

This means that the net yield of thyroxine, calculated on the di-iodotyrosine destroyed, was 1·5%; the gross yield was 0·33%. von Mutzenbecher records a gross yield of 0·24%; he does not state the amount of di-iodotyrosine recovered unchanged so that his net yield cannot be calculated. Block (1940) and Barkdoll & Ross (1944), working under conditions similar to ours, obtained gross yields of 0·12 and 0·2–0·35% respectively.

Effect of thiosulphate. An experiment on the lines just described but starting at the more favourable initial pH of 10 (cf. Table 1) was carried out in presence of sodium thiosulphate equivalent in amount to the iodine content of the di-iodotyrosine used. The period of incubation was reduced in this case to 9 days and a control experiment was run simultaneously for the same period under the ordinary conditions; the amount of di-iodotyrosine used in each experiment was 9·4 g. of the dihydrate. In the control the thyroxine isolated amounted to 46 mg. and in the thiosulphate experiment to 1 mg.; allowing for the recovery of unchanged di-iodotyrosine these figures correspond to net yields of 1·6 and 0·04% respectively; it is apparent therefore that the presence of thiosulphate almost entirely suppresses the formation of thyroxine.

Effect of exclusion of oxygen. In the preceding experiment a third similar quantity of di-iodotyrosine was incubated for the same length of time under completely anaerobic conditions (McIntosh-Fildes jar). No thyroxine could be isolated and 92% of the di-iodotyrosine was recovered unchanged.

Experiments with added oxidizing agents

(1) *Potassium ferricyanide.* Oxidation of di-iodotyrosine in alkaline solution with potassium ferricyanide under the conditions employed by Pummerer and his co-workers (1922, 1925) for the oxidation of *p*-cresol led only to the formation of intractable tarry products of acidic nature. An attempt was made to apply the butanol technique subsequently successfully used with peroxide (see below).

Di-iodotyrosine dihydrate (11·75 g.) was dissolved in NaOH (47 ml. of 1·015 N; 95% neutralization) and 3 ml. water and the solution was chilled in ice. *n*-Butanol (50 ml.) was added and the mixture, continuously shaken in an ice-bath, was treated with a solution of potassium ferricyanide (8·22 g.) in water (25 ml.) added over 30 min. The mixture was shaken at room temperature for an hour and the layers were separated; the aqueous solution was re-adjusted to pH about 8·5 (20 ml. of N-NaOH required); more butanol (100 ml.) was added and the oxidation continued at 0° with a further 8·22 g. of ferricyanide in 25 ml. water, the reaction being kept alkaline by periodic addition of NaOH (total 30 ml. N solution). The butanol extracts were washed with N-NaOH and evaporated. The residue was taken up in water and precipitated at the boiling point with HCl. The material thus obtained, after drying, was almost completely soluble in ether; no thyroxine could be isolated from the small ether-insoluble residue, nor was any di-iodotyrosine recoverable from the original aqueous solution.

(2) *Hydrogen peroxide at* 37°. An experiment typical of those recorded in Table 2 was as follows. Di-iodotyrosine dihydrate (18·8 g.) was dissolved in N-NaOH (76 ml.; 95% neutralization) to give a solution having pH about 10. Hydrogen peroxide (0·5 atomic equiv. O) was added and the mixture was incubated at 38°. After 9 hr. very little peroxide was detectable. After about 16 hr. the solution was worked up as described under the simple incubation experiments. The thyroxine isolated amounted to 36 mg. and the di-iodotyrosine recovered to 3·52 g.; the net yield of thyroxine was thus 1·5% and the gross yield 0·23%.

(3) *Hydrogen peroxide in excess at higher temperature* (Table 3A). Di-iodotyrosine dihydrate (9·4 g.) was dissolved in N-NaOH (38 ml.) and the solution was heated on the boiling water-bath. Hydrogen peroxide (20 vol. solution; 5 atomic equiv. O in all) was added in fifteen portions over about 1½ hr.; the reaction was maintained alkaline to phenolphthalein throughout. The solution, which had become very dark, was worked up as usual. Of the di-iodotyrosine 54% was recovered; the thyroxine isolated was 58 mg. (1·6% net yield; 0·6% gross).

(4) *Hydrogen peroxide in excess at high temperature with butanol* (Table 3B). The most successful experiment by this technique, the rationale of which has been discussed in the introductory portion of the paper, was the following.

Di-iodotyrosine dihydrate (23·5 g.) was dissolved in N-NaOH (95 ml.); 100 ml. of *n*-butanol, previously saturated with water, were added and the mixture was heated on the boiling water-bath. Additions of 10 ml. portions of 20 vol. hydrogen peroxide were then made, with continued heating and vigorous shaking, at intervals, such that peroxide did not accumulate in the solution. After each five additions

of peroxide the butanol layer was separated and replaced with fresh solvent, the amounts being increased in approximate parallelism with the increase in volume of the aqueous layer; the pH of the latter was readjusted to approximately 10 at frequent intervals during the experiment by addition of the requisite amount of N-NaOH. In all, 75 additions of hydrogen peroxide were made, i.e. approximately 25 atomic equivalents of oxygen/mol. of di-iodotyrosine; the entire procedure occupied about 5 hr. In the early stages the butanol extracts were fairly highly coloured, but the last few were almost colourless.

The combined butanol extracts were concentrated under diminished pressure to 1500 ml. and extracted once with 1000 ml. of N-NaOH; the latter extract was washed with 500 ml. of butanol and this washing, together with the main butanol solution, was evaporated to dryness. The residue was taken up in water (400 ml.) and after removal of traces of butanol by a brief vacuum distillation the solution was brought to the boil and acidified (about pH 4) with dilute H_2SO_4. Next day the precipitate was collected and dried in a vacuum desiccator. The dried material (1·55 g.) was ground up with dry ether and collected, this process being repeated three times; the last ethereal extract was almost colourless and the insoluble residue amounted to 0·87 g. This was dissolved in the minimum amount (64 ml.) of boiling 0·1 N-K_2CO_3; after 22 hr. at 0° the precipitate of potassium salt was collected and converted into thyroxine in the usual manner by solution in alkaline aqueous ethanol followed by precipitation at the boiling point with acetic acid; the pure crystalline thyroxine thus obtained amounted to 278 mg. Further crops of thyroxine, amounting in all to 45 mg., were isolated by collecting all the aqueous acid-insoluble material from the mother liquors of the potassium salt and of the thyroxine crystallization and purifying it by repeating the extraction from alkaline solution into butanol and separation as potassium salt. The total yield of thyroxine was thus 323 mg.

The original aqueous solution was combined with the alkaline washing of the butanol extracts and concentrated somewhat under diminished pressure; it was then brought to the boil, acidified strongly with concentrated HCl and filtered hot from a tarry precipitate; the filtrate was adjusted to pH 4 by addition of 10 N-NaOH and the di-iodotyrosine which separated on cooling was collected (10·66 g.). Two further crops of di-iodotyrosine were obtained by concentration of the mother liquor, the total amount recovered being 13·71 g.

The di-iodotyrosine which had been oxidized was therefore 9·79 g. and the net yield of thyroxine was 4%.

The thyroxine which was isolated in this and other experiments crystallized in the characteristic sheaves of bent needles and had m.p. 240° (decomp.); it gave a positive ninhydrin reaction and a clear red colour after heating with nitrite and HCl in ethanol followed by treatment with excess of ammonia. The product obtained in the experiment just described was completely analyzed with the following results. Found: C, 23·5; H, 1·7; N, 1·8; I, 65·3%. (Calc. for $C_{15}H_{11}O_4NI_4$: C, 23·2; H, 1·4; N, 1·8; I, 65·4%.) It also showed activity in the tadpole metamorphosis test equal to that of authentic thyroxine.*

(5) *Iodine.* Di-iodotyrosine dihydrate (9·4 g.) was dissolved in N-NaOH (38 ml.); iodine (2·65 ml. of 3·78 N solution; 0·5 atomic equiv. O) was added, followed by *n*-butanol (60 ml.). The whole was shaken for 3 hr. at 37° after which no free iodine could be detected; the aqueous layer was then made N with respect to NaOH, the butanol was separated and, together with a second similar extract, worked up as usual; 15 mg. of thyroxine were obtained, whilst 72% of the di-iodotyrosine was recovered from the aqueous solution. The net yield of thyroxine was therefore 0·4%.

Optical activity of l(+)-di-iodotyrosine and l(−)-thyroxine

The di-iodotyrosine which was used in the experiments described in this paper was prepared from optically pure *l*-(−)-tyrosine having $[\alpha]_D^{22°} = -12·4°$ in 5% solution in N-HCl; the iodination was carried out either by the method of Harington (1928) or that of Block & Powell (1943) and in all cases the product had $[\alpha]_D^{22°} = +1·5°$ in N-HCl, the concentration being about 5%. This rotation is considerably lower than that previously recorded (cf. Abderhalden & Guggenheim, 1908; Harington, 1928), but our material was undoubtedly optically pure, since on catalytic deiodination it gave a 94% yield of tyrosine having $[\alpha]_D^{22°} = -12·2°$ in N-HCl. We feel therefore that +1·5° must be regarded as the correct figure for the rotation of *l*(+)-di-iodotyrosine under the prescribed conditions, although we can offer no explanation of the higher figures previously recorded.

There is no reason to suppose that any racemization is involved in the oxidative conversion of di-iodotyrosine into thyroxine, so that the rotation of the thyroxine isolated in our present experiments should be that of the optically-pure natural substance. We have found for our preparation $[\alpha]_D^{22°} = -4·3°$ ($c = 3·03$ in a solution made up of 24 g. 0·5 N-sodium hydroxide and 56 g. of ethanol); this compares with $[\alpha]_D = -4·4°$ observed by Foster, Palmer & Leland (1936) under the same conditions for a sample of thyroxine isolated from thyroid by acid hydrolysis. The figure now obtained is larger than that observed by Harington (1928) for *l*(−)-thyroxine prepared by resolution of *dl*-3:5-di-iodothyronine and subsequent iodination; it must be concluded that this resolution was not quite complete. It may be noted that the rotation of thyroxine, like that of other amino-acids, is much influenced by the nature of the solvent; thus our present preparation, dissolved at a concentration of 3·28% in a mixture of 1 vol. N-NaOH and 2 vol. ethanol, had $[\alpha]_D^{21°} = -5·04°$.

* We are indebted to Dr R. Deanesly for carrying out this test.

Intrinsic instability of di-iodotyrosine (see Fig. 1)

Quantities of 470 mg. (1 mmol.) of di-iodotyrosine dihydrate were dissolved rapidly by boiling in 25 ml. water containing the requisite amount of HCl or NaOH to give the desired pH. The solutions were then heated for 1 hr. on a boiling water-bath, cooled, treated with 12·5 N-NaOH to make a final concentration of N and extracted once with 25 ml. of butanol. The aqueous layer was separated quantitatively and submitted to a brief vacuum distillation to remove butanol; it was then transferred to a boiling tube, being made up to 30 ml. with washings, and acidified when hot with 3 ml. glacial acetic acid.

At the end of the period of heating a great difference in the behaviour of di-iodotyrosine at the various pH values was already apparent; the solutions in the pH range 6–9 became dark brown, and at the stage of acidification threw down a dark-coloured amorphous precipitate which was removed by filtration before crystallization of residual di-iodotyrosine set in. In all cases crystallization was completed by keeping in the ice-chest overnight; the residues of unchanged di-iodotyrosine were then collected, dried *in vacuo* over P_2O_5 and weighed.

SUMMARY

1. The work of von Mutzenbecher (1939) on the formation of thyroxine by incubation of di-iodotyrosine in alkaline solution is confirmed. It is shown that this conversion is prevented by exclusion of atmospheric oxygen or by addition of thiosulphate to the solution; the reaction is also profoundly influenced by the pH during incubation.

2. The effect of added oxidizing agents on the conversion of di-iodotyrosine to thyroxine has been studied; with the aid of hydrogen peroxide increased yields of thyroxine can be obtained in a much shorter time, the effect of pH being similar to that observed on simple aerobic incubation. The conversion can also be accelerated by addition of iodine. Reasons are given for supposing that in all cases observed the actual agent effective in bringing about the oxidation of di-iodotyrosine to thyroxine is free iodine, and attention is called to the biological implications of this hypothesis.

3. The thyroxine obtained by oxidation of *l*(+)-di-iodotyrosine, itself derived from *l*(−)-tyrosine, is shown to have an optical rotation identical with that of the natural product; this provides direct chemical proof of the configurative relationship of natural tyrosine and natural thyroxine.

4. Di-iodotyrosine in aqueous solution has been found to exhibit a remarkable degree of instability towards heat over the pH range 4–9; at higher acidity or alkalinity the amino-acid is comparatively stable.

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(*An Addendum has been added on p.* 212.)

Note on the Nutritive Value of the Nitrogenous Substances contained in Dried Yeast (*Torulopsis lipofera*)

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The yeast was a dried specimen of *Torulopsis lipofera*, a species having a high fat content, provided by Dr A. Kleinzeller (1944). Its composition was: moisture less than 1, fat 18·6, nitrogen 5·85, 'crude' protein (N × 6) 35·1%. Analyses by Stutzer's method showed that about 10% of the nitrogen was non-protein in character.

The following experiments were designed to show the nutritive value of the nitrogenous substances, protein and non-protein, contained in the yeast, as

measured by their capacity to support growth in young rats, and the supplementary value, if any, possessed by these nitrogenous substances for the proteins contained in wheat flour.

EXPERIMENTAL

The young rats (Lister Institute strain of pied rats) of average weight 52–54 g. received the experimental diets a few days after weaning. The diets were arranged to be adequate in all respects except for their content of protein, which was suboptimal and was the factor limiting growth. The rate of weight increase of litter-mates was compared on the following three diets, all of which contained about 2·1% nitrogen or 12–13% crude protein (calculated on a dry-weight basis). The nitrogen in diet 1 was derived entirely from whole-wheat flour (representing 100% of the grain); of that in diet 2 about three-quarters was derived from whole-wheat flour and about one-quarter from the yeast; that in diet 3 was derived entirely from the yeast. The composition of the diets is shown in Table 1. Diet 1 contained 7 parts of fat as hardened arachis oil, diet 2 only

Table 1. *Composition of the diets used*

	Parts by weight		
	Diet 1	Diet 2	Diet 3
Whole-wheat flour	107	80	—
Yeast	—	10·3	38
Pure maize starch	—	17	69
Hardened arachis oil	7	5·1*	—†
Salt mixture (McCollum no. 185)	2	2	2
Cod-liver oil	1	1	1
Average N content	2·14	2·12	2·08
Average protein content‡ (% of dry matter)	12·8	12·7	12·5

* 1·9 parts of fat supplied by the yeast.
† 7·1 parts of fat supplied by the yeast.
‡ N × 6.

5·1 parts and diet 3 none, to make allowance for the fat supplied by the yeast in these two diets. Each diet contained 2 parts of McCollum's no. 185 salt mixture to correct any mineral deficiency and 1 part of cod-liver oil to provide vitamins A and D. A daily ration of 1 ml. of a protein-free yeast extract (equivalent to 0·5 g. dried brewers' yeast) was given separately to each rat to provide an excess of B vitamins.

The tests lasted for 6 weeks. During the fourth and fifth weeks of each experiment, measurements were made of the intake of food and of the weight of faeces passed and of their nitrogen content. From these results calculations could be made of the coefficient of (apparent) digestibility of the protein in each diet, as well as of the ratio of body-weight increase to g. protein eaten, the ratio adopted by Osborne, Mendel & Ferry (1919) to express nutritive value of protein. These figures are set out in Table 2.

RESULTS

At the end of 6 weeks, the rats receiving the wheat-flour diet 1 reached an average body weight of 127 g., while the average weight of those on the

Table 2. *Protein utilization by rats receiving diets* 1, 2 *and* 3

Period of test = 6 weeks.

Diet	Source of protein	Protein* content of diet (% of dry matter)	No. of rats	Mean body weight (g.)		Average weekly†						Coefficient‡ of digestibility of N	Growth-promoting value§ of protein	Food eaten/unit weight increase (g. dry matter/g.)
				Initial	Final	Weight increase (g.)	Food intake (g. of dry matter)	N intake (g.)	Protein* intake (g.)	Faeces passed (g. of dry matter)	N in faeces (g.)	N digested (g.)		
1	Whole-wheat flour	12·8	5	52	127	11·8	64·6	1·37	8·22	6·70	0·26	1·11	81·1	1·44	5·5
2	75% from whole-wheat flour; 25% from yeast	12·7	6	54	133	13·2	64·9	1·38	8·28	6·79	0·29	1·08	78·6	1·59	4·9
3	Yeast	12·5	5	54	85	5·3	45·7	0·94	5·64	5·53	0·21	0·74	78·0	0·94	8·6

* N × 6.
† Amounts per rat, from estimations made during weeks 4 and 5.
‡ $100\left(\frac{\text{Nitrogen intake} - \text{nitrogen in faeces}}{\text{Nitrogen intake}}\right)$.
§ g. wt. increase/g. protein eaten (Osborne *et al.* 1919).

yeast diet 3 was only 85 g.; those on diet 2, in which yeast and whole-wheat flour were combined, were slightly heavier than those on diet 1, the average body weight being 133 g.

The rats receiving diets 1 and 2 ate more food than those on diet 3 and made better use of it; the intake of dry food corresponding to 1 g. increase in body weight was respectively 5·5, 4·9 and 8·6 g. The nutritive values of the proteins in the three diets, measured by the weight increase per g. protein eaten (Osborne *et al.* 1919) were in a corresponding order, the figures being respectively, 1·44, 1·59 and 0·94. The digestibility coefficient calculated for the proteins in the three diets 81, 79 and 78, did not differ materially from one another.

These figures show that, whereas the proteins contained in the yeast were much less efficient for support of growth of young rats than those contained in whole wheat, yet a mixture of the two in a diet, in the proportion of 1 part of yeast proteins to 3 parts of wheat proteins, had a nutritive value as great as, possibly slightly greater than, that of whole wheat (see Fig. 1). In other words, the mixture of amino-acids in the proteins of the yeast had a marked supplementary effect for those contained in whole wheat.

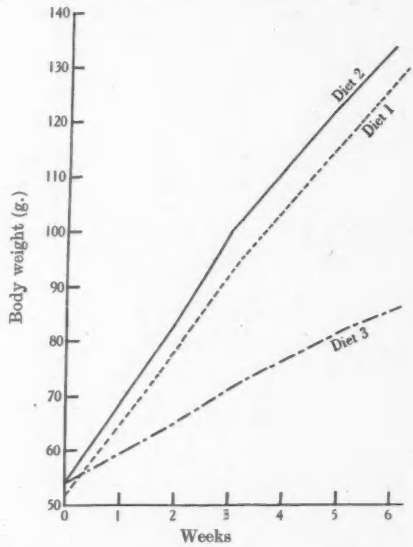

Fig. 1. Average growth curves of 5–6 young rats receiving: ----- diet 1, protein derived from whole-wheat flour; —— diet 2, 75% of protein derived from whole-wheat flour, and 25% from yeast; – – – – diet 3, protein derived from yeast. All diets contained about 13% 'crude' protein (on a dry-weight basis).

This result confirms the results of many previous workers who have found that yeast proteins, though possessing a relatively inferior nutritive value when given as sole source of nitrogen in a diet (Still & Koch, 1928; Klose & Tevold, 1944), yet, when combined with those of cereals, form a mixture which enhances the value of the latter (Light & Frey, 1943; Macrae, El Sadr & Sellers, 1942; Kon & Markuse, 1931).

Klose & Tevold (1944) traced the nutritive defect in yeast proteins to a deficiency of the essential amino-acid methionine. A few years earlier, Daft, Sebrell & Lillie (1941) had shown that the necrosis of the liver in rats fed on diets containing only 4% casein could be prevented by increasing the proportion of casein to 30% of the diet or the methionine content of the diet by an amount (0·7%) corresponding to that of the additional casein. Recently, Himsworth & Glynn (1944 *a*, *b*) have found that liver necrosis occurs in rats if the protein of the diet is derived from bakers' yeast (*Saccharomyces cerevisiae*), and the content of 'crude' protein is 7% or even higher. This necrosis could be prevented by adding 8% of casein, or the equivalent amount of methionine, to the diet.

We also have observed severe necrosis and fatty degeneration of the liver of rats fed on diets containing 4–6% casein as source of protein but, in the present series of experiments, no histological lesions were found in the liver of a rat which had been fed on diet 3 for 40 days. After 80 days there was apparent a certain degree of degeneration, limited to the cells adjacent to the portal areas; the degree of abnormality was, however, slight. In diet 3 the proportion of protein in the diet, 13% of the dry weight, was relatively high, and it is also possible that the proteins of *Torulopsis lipofera* are less deficient in methionine than those of the species of yeast used by Himsworth & Glynn.

The proteins contained in wheat and other cereals, compared with those of yeast, are relatively rich in the sulphur-containing amino-acids methionine and cystine, and poor in lysine, an amino-acid essential for growth. The proteins of brewers' yeast, and presumably of other types of yeast also, while relatively poor in methionine, are well supplied with lysine. These facts offer an explanation of the complementary action shown by combinations of these two classes of proteins for maintenance of growth in young animals.

SUMMARY

1. The nitrogenous substances in the yeast *Torulopsis lipofera*, when employed as sole source of nitrogen in an otherwise complete diet, supported growth of young rats at a rate much inferior to that of litter-mates on a diet containing the same proportion of nitrogen derived from whole-wheat flour.

2. When, however, one-quarter of the nitrogen in the diet was derived from the yeast and three-quarters from whole-wheat flour, the rate of weight increase and utilization of nitrogen was slightly greater than when all the nitrogen was obtained from the whole-wheat flour.

3. This complementary action is presumably due to the relatively high proportion in yeast protein of lysine, an amino-acid essential for growth, which is present in cereal proteins in relatively low amount.

We wish to thank Sir Charles Martin, who kindly examined the histological specimens, and Mr T. C. Fletcher, who kindly prepared them. Our thanks are also due to Mr G. W. Flynn for his help in the preparation of the diets and in the care of the experimental animals.

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The Mechanism of Polysaccharide Production from Sucrose. 2

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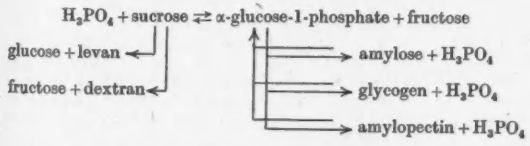
Different polysaccharides can be produced from either α-glucose-1-phosphate or sucrose by the action of cell-free enzymes. From α-glucose-1-phosphate there have been obtained amylose, amylopectin, and glycogen (Hanes, 1940; Hassid, Cori & McReady, 1943; Haworth, Peat & Browne, 1944; Cori & Cori, 1943), whereas from sucrose the formation of a glucosan, dextran (Hehre & Sugg, 1942; Hehre, 1943) and a fructosan, levan (Hestrin, Avineri-Shapiro & Aschner, 1943) have been reported.

There is a structural similarity between the two substrates which are employed for these polymerizations. The relation of the substrates has been emphasized further by the recent demonstration that α-glucose-1-phosphate and fructose enter into dynamic equilibrium with sucrose and phosphoric acid in the presence of an enzyme which has been isolated from a suitable bacterial species (Doudoroff, 1943; Hassid, Doudoroff & Barker, 1944; Doudoroff, Hassid & Barker, 1944). The known syntheses of different polysaccharides can, in the light of these observations, be fitted into a single scheme:



The group name *polymero-synthease* provides us with a generic description of the enzyme entities which mediate the polymeric steps of the scheme presented above. The elements of water are not included in the balance sheet of the listed reactions. The action of the *polymero-syntheases* on their substrates can therefore be described as a *direct polymeric degradation* (Leibowitz & Hestrin, 1945). *Levansucrase* is the name which has been given to the polymero-synthease which degrades suitable aldosido-fructosides (sucrose, raffinose) polymerically into aldose (glucose, melibiose) and levan.

Levansucrase has been obtained in cell-free solution in concentrations suitable for kinetic studies from an organism which has been designated as *Aerobacter levanicum* (Hestrin *et al.* 1943).

Our levansucrase preparation catalyzes the following transformation (Hestrin & Avineri-Shapiro, 1943, 1944):

$$(n+m)\ \text{sucrose} + (n)\ H_2O \rightarrow (n)\ \text{invert sugar} + (m)\ \text{glucose} + (\text{levan}), \quad (1)$$

m and n being molar quantities. The action of the preparation is thus two-fold, hydrolytic and polymeric. Elsewhere evidence has been advanced which shows that the polymeric reaction (m-term of equation (1)) is not a sequence of the hydrolytic reaction (n-term of equation (1)) (Hestrin, 1944). The present paper defines the conditions of pH, substrate concentration, enzyme concentration and reaction time in which levansucrase acts with maximum efficiency in terms of production of polymer.

It is to be assumed that information concerning

the activity of levansucrase with respect to environmental influences will help to shed light on many unknown features of the action of this enzyme, including the following: (*a*) the possible interconnexion of the polymeric and hydrolytic activities of the levansucrase preparation, (*b*) the possibility of obtaining a reversal of the reaction, (*c*) the possibility that the polymeric degradation is effected by a single enzyme entity, and (*d*) the question raised in a general form by Stacey (1943) of the possible participation of polymero-synthease constituents in the lattice of the formed polymer.

An added interest was further given to the study of details of the process of levan production by the fact that levan, alone among the known enzyme-synthesized polysaccharides, consists of fructofuranosidic rather than glucopyranosidic residues. Moreover, the particle weight of levan, as measured by Ingelman & Siegbahn (1944), seems to be unusually high, approaching and even exceeding in magnitude such biological units as viruses and genes.

METHODS

The preparation of the enzyme and the analytical methods have been described (Hestrin *et al.* 1943; Hestrin & Avineri-Shapiro, 1944). The source of the enzyme is the organism *Aerobacter levanicum* which is obtainable from the Department of Hygiene and Bacteriology of the Hebrew University, Jerusalem. The reaction mixtures were maintained at 37°. Determinations of levan were carried out on volumes of reaction mixture sufficient to permit analytical accuracy within a range of error of 3% of the reading obtained. Sterility was maintained by the addition of a drop of chloroform containing thymol to the test mixtures. Free fructose was estimated by subtracting from the total of the reducing sugar the hexose equivalent of the amount of sugar bound as levan, and dividing the result by two. The validity of this method of computation has been demonstrated (Hestrin & Avineri-Shapiro, 1944).

RESULTS

Effect of pH

Solutions of 3 ml. contained 1 ml. of McIlvaine phosphate-citrate mixture of designated pH, 1 ml. of 15% sucrose in water, and 1 ml. of enzyme. Reaction was interrupted after 5 hr. At the time of analysis levan was still being actively formed in all mixtures whose pH permitted levan production.

A typical experiment, set forth in Fig. 1, shows that the pH-activity curve of levansucrase is regular and symmetrical and that there is a well-marked optimum in the region of pH 5·0–5·8. Fructose production from sucrose by the same levansucrase preparation was also optimal in the same region. The pH-activity curve of dextransucrase as established by Hehre & Sugg (1942) shows remarkable similarity to the pH-activity curve of levansucrase.

The pH-activity curve of levansucrase is definitely monophasic. Hence it presents no special support for the view that more than one enzyme system in the preparation effects a production of levan from sucrose.

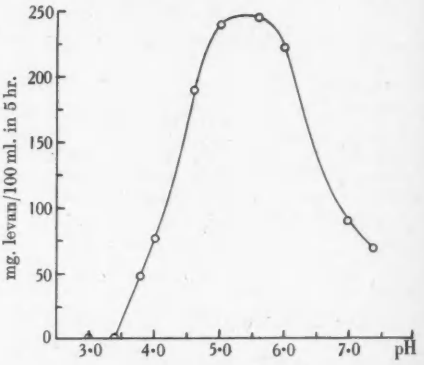


Fig. 1. Effect of pH.

Sucrose concentration. Effect on rate of reaction

The test mixtures contained graded concentrations of sucrose, 1 ml. of enzyme solution, and 1 ml. of Sorensen citrate buffer pH 5·0 in a total volume of 3 ml. Reaction was interrupted after 3 hr. At this time, synthesis was less than 50% complete.

The results, presented in Table 1, show a typical picture: a progressively smaller increase in rate of reaction as the substrate concentration was raised to about 5% sucrose, and constancy or a slow decrease in rate of reaction as sucrose was increased beyond this optimum concentration. The concentration of sucrose which permitted half-optimum reaction velocity varied with the preparation of enzyme. The molar magnitudes were considerably greater than the corresponding value for the hydrolysis of glucose-1-phosphate by muscle phosphorylase, and of the same order as the corresponding values frequently recorded for sucrase of yeast. The data show fair conformity with the Michaelis-Menten theory, but are inadequate as a basis for any definite statement on this point.

Table 1. *Variation of rate of reaction with concentration of sucrose*

Sucrose (%)	Levan (mg./100 ml.) First enzyme sample	Second enzyme sample
0·5	45	20
1·0	64	—
2·0	95	58
3·0	—	84
5·0	117	102
10·0	108	84

Sucrose concentration. Its effect on the final yield of levan

The final yields of levan were determined in test mixtures of similar composition but incubated for 48 hr. Longer incubation of the mixtures failed to bring about any material increase of the levan yield. The results are set forth in Fig. 2.

At substrate concentrations below 3% sucrose there was marked increase in the absolute yield of levan with increasing sucrose concentration. But when the sucrose concentration was increased beyond 3% no further noteworthy increase in the absolute yield of levan was gained. The percentage yield of levan, calculated on total fructose originally present bound in sucrose, shows an opposite trend: it rises with decreasing sucrose concentration, this rise being slow between 18 and 3% sucrose, but increasingly rapid with diminishing substrate concentration below 3% sucrose. Extrapolation of the curve suggests that the levan yield calculated on total fructose residues would be greater than 75% of theory at an infinitely low concentration of sucrose. On 0·5% sucrose, a levan yield amounting to 62% of theory was in fact obtained. We shall later consider some of the factors which shape this curve.

The data presented in Fig. 2 cannot be used for calculating the equilibrium constant of the reaction of levan synthesis even if such a constant exists. Levan yield with high sucrose concentrations (>5%) being independent of the sucrose concentration, it is evident that levan values obtained in this range are not determined by an equilibrium relation between sucrose and levan. Again, levan values on low sucrose concentrations (<3%) are evidently determined by the amount of sucrose which was originally available. However, they cannot be used for the calculation of any equilibrium constant in the absence of parallel data indicating the extent of the hydrolytic breakdown of sucrose effected in the same conditions by the enzyme, and the nature of the relation between the polymerative and the hydrolytic breakdown processes. Nevertheless, the data available are sufficient to establish qualitatively that the ratio of levan to sucrose at the true equilibrium, if there is such a point, is considerably higher than 1/1.

Sucrose concentration. Effect on the levan/fructose ratio

An orienting experiment on the relation between levan and fructose production at varying concentrations of substrate is presented in Fig. 2*a*. Test mixtures were composed in the same manner as for the experiment of Fig. 2*b*. Reaction was interrupted after 5 hr. At this time, synthesis was nearing a standstill in mixtures containing <3% sucrose. Longer incubation was avoided as it is known to favour production of fructose relative to levan (Hestrin *et al.* 1943).

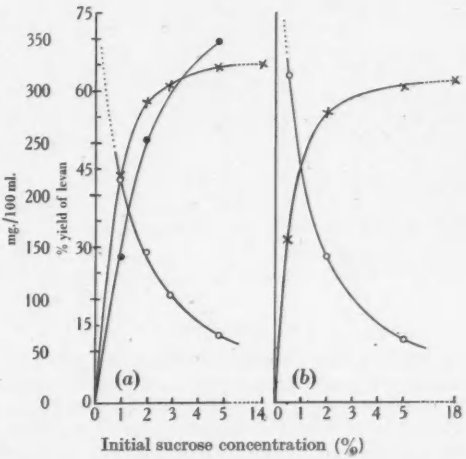


Fig. 2. Enzyme activity in prolonged incubation times as a function of sucrose concentration. o, percentage levan yield calculated on total fructoside supply; ×, levan in mg./100 ml.; •, free fructose in mg./100 ml. Incubation time: (*a*) 48 hr.; (*b*) 5 hr.

It may be seen that low sucrose concentrations tend to favour levan synthesis at the expense of fructose production. A similar conclusion also follows from comparison of the present figure with an earlier balance sheet analysis which showed the reaction undergone by 2% sucrose (Hestrin & Avineri-Shapiro, 1944). The observation suggests that relative diminution of fructose production is a partial explanation of the accelerated rise in the percentage yield of levan observed as the sucrose concentration is diminished.

Enzyme concentration. Effect on specific activity

Experiments showing the variation of specific enzyme activity with the enzyme concentration are presented in Table 2. The data reveal a sharp drop in specific activity as the enzyme concentration is raised, independently of whether activity is measured in terms of levan production or in terms of the total sucrose amounts decomposed. This is a property which, if it is a general one, differentiates levansucrase from yeast sucrase or phosphorylase, both of which show nearly constant specific activity throughout a wide range of concentration.

Considerable variation in activity between different lots of enzyme has been noted. The variation is due largely to the fact that in the method used extraction of levansucrase in soluble form from the

Table 2. *Variation of rate of reaction with concentration of enzyme*

(1 ml. of Sorensen citrate buffer pH 5·0, 1 ml. of 15% sucrose solution and varying amounts of enzyme in a total volume of 3 ml.; incubation times: 2 hr. for enzyme A, and 3 hr. for enzyme B.)

Enzyme preparation	Vol. of enzyme (ml.) (x)	Levan (mg./100 ml.) (m)	Fructose (mg./100 ml.) (n)	Specific enzyme activity (m/x)	Specific enzyme activity $(m-n)/(x)$
A	0·2	97	—	485	—
	0·4	138	—	345	—
	0·6	142	—	237	—
	0·8	145	—	181	—
	1·0	156	—	156	—
B	0·3	68	—	227	—
	0·5	112	76	224	376
	0·8	144	94	180	297
	1·0	143	101	143	244

Aerobacter material is never complete. Under the conditions of the preparation, young cells autolyzed in thick suspension consistently yield an active solution. But old cells (48 hr. culture) retain the enzyme more tenaciously; and even young cells fail to release the enzyme if their autolysis is carried out in a dilute suspension.

In comparing the activity of different samples of the bacterial enzyme, the following unit has been found useful. A Levansucrase Unit (L.U.) is defined as that minimum amount of enzyme which induces production of 100 mg. of levan/100 ml. in reaction mixtures of 3 ml. containing 5% of sucrose at pH 5·0 after incubation for 2 hr. at 37°. This definition obviates the complication introduced by the variation of the specific activity of levansucrase with the enzyme concentration and gives the enzyme activity in terms which correspond to measurements carried out in conditions optimal for synthesis. As levan production initially follows a practically linear course under the conditions selected, resort to linear extrapolation in estimating L.U. is permissible so long as the levan concentration actually measured does not deviate from 100 mg./100 ml. by more than about 50%. Using a turbidity equivalent of this amount of levan for reference, observation of this proviso offers no difficulty in practice. The most active of our cell extracts (Preparation A, Table 2) rated 5 L.U./ml.

Enzyme concentration. Effect on the final yield of levan

Mixtures contained graded concentrations of enzyme in a total of 3 ml. fluid containing 5% of sucrose buffered at pH 5·0 with 1 ml. of Sorensen sodium citrate solution.

A graphic presentation of the results is given in Fig. 3. It is seen that enzyme concentration under the conditions of the experiment determines levan yield where small amounts of enzyme are employed. When a sufficiency of enzyme is initially added, however, further increase in the enzyme concentration does not bring about any further increase in the yield.

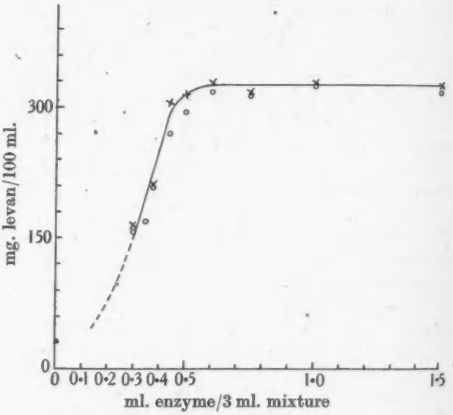


Fig. 3. Effect of enzyme concentration on levan yield. o, levan after 48 hr. incubation; ×, levan after 72 hr. incubation.

Two explanations of the rising sector of the curve may be mentioned: (1) in low concentration the enzyme is relatively unstable and limits the levan yield because it is thermally inactivated in the course of the incubation; (2) polymer production involves a stoichiometric inclusion of some enzyme element within the molecule of the polymer, and levan yield becomes dependent on this factor when the concentration of the latter becomes a limiting one.

The flat sector of the curve in conjunction with the data presented in Fig. 2*b* shows that conditions can be realized experimentally in which the final absolute yield of levan stays constant despite large variation in the enzyme and sucrose supply. Under these conditions some unidentified factor, which is neither the initial sucrose nor enzyme supply but may possibly be the accumulation of a reaction product, puts a stop to levan synthesis long before the supply of sucrose has been exhausted.

Time relations

The time course of the production of levan by the action of levansucrase has been investigated for the condition of non-limiting sucrose concentrations. The test mixtures contained 5% of sucrose and 1 ml. of enzyme liquid (or suspension) in a total volume of 3 ml. and were buffered at pH 5·0.

Results pertaining to several lots of soluble enzyme and one sample of desmo-enzyme (cell sediment of an autolysate) are assembled in Fig. 4 and its inset.

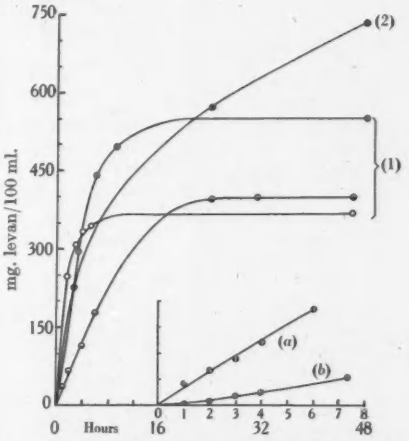


Fig. 4. Kinetics of levan production *in vitro*. Curves, (1) levan production by different soluble levansucrase preparations; (2) by an insoluble levansucrase preparation. Inset, (*a*) soluble enzyme without added glucose; (*b*) the same with 4% added glucose.

Levan production in the condition investigated is seen to follow initially a practically linear course. This is maintained both in the absence and presence of added glucose during a period of 3 hr. or until 150 mg. of levan/100 ml. have formed, whichever is the shorter. There is no sign here of an autocatalytic trend such as has been noted in glycogen production *in vitro*. Since no decrease in reaction rate takes place over a considerable interval it has to be concluded that no rate-limiting component is being used up during this time. Neither the amount of glucose, which inhibits markedly when sufficiently concentrated relative to sucrose (see Fig. 4 inset and Hestrin & Avineri-Shapiro, 1944), nor the fructose nor levan formed at this stage, exerts any notable influence on the early course of the synthesis.

The time-action curves clearly reveal the intervention of a sharply inhibiting influence in a late phase of the synthetic process. As a limiting concentration of levan is approached, the curves for soluble levansucrase bend rapidly and flatten. The limiting levan concentration, variable to some extent with the particular preparation of enzyme, is quite low (< 500 mg./100 ml.). It has been shown to be independent of the sucrose and enzyme concentrations (cf. Figs. 2*b*, 3). No similar limit point is discernible when the synthesis is mediated by desmo-levansucrase (cf. Fig. 4).

When a large excess of yeast invertase is incorporated in the same test mixtures together with levansucrase, the final amount of levan formed is smaller than in mixtures without added yeast sucrase. This effect is certainly due to the removal of sucrose from the system at an early stage by the vigorous action of yeast invertase. It is significant that the removal of sucrose by this agent does not lead to a reversal of the direction of the levansucrase action which would express itself in a removal of already formed levan.

SUMMARY

1. The activity of the levansucrase of *Aerobacter levanicum* has been investigated as to dependence on pH, sucrose concentration and enzyme concentration. Levan synthesis was found to proceed at an optimum rate in mixtures containing 5% of sucrose buffered at pH 5·0–5·8. A levansucrase unit which expresses the enzyme activity in optimum reaction conditions has been defined on this basis.

In estimating levansucrase activity, consideration must be given to the factor of the specific enzyme activity. This decreases sharply as the enzyme concentration is raised.

2. The time-action curve of soluble levansucrase in the presence of a non-limiting concentration of sucrose and a sufficiency of enzyme follows initially a practically linear course, but later bends sharply and flattens as an unidentified inhibiting influence comes into play.

3. The final absolute levan yield is a function of the initial sucrose and enzyme supply when low concentrations of substrate or enzyme are employed, but becomes independent of either of these factors when the latter are increased sufficiently.

On a suitably low sucrose concentration, levan production accounts finally for 62% of the total of the fructoside residues. The levan/sucrose ratio at the true equilibrium point is therefore certainly greater than 1.

The authors wish to acknowledge their indebtedness to Dr M. Aschner and Dr J. Leibowitz for their interest in this work.

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Acid-producing Mechanisms in Minced Leaves

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(*Received* 5 *February* 1945)

Bawden & Pirie (1944) observed that the pH of a suspension of washed minced tomato leaves, neutralized with NaOH, drifted back towards the original value of pH 5·8–6·2. By making further additions of alkali during 24 hr. the pH could be finally stabilized at 7·6–8·0. A similar drift has been found in neutralized tobacco and bean-leaf suspensions. The present investigation was undertaken to find the cause of this pH drift. There appeared to be three possibilities: the alkaline hydrolysis of an ester giving rise to an acid, the enzymic hydrolysis of an ester taking place when the pH was raised, or the metabolic production of carbon dioxide. Neuberg & Ottenstein (1928) described the enzymic demethylation of pectin in minced tobacco leaves in the presence of sap. They worked at the normal pH of the mince, whereas the pH drift with which this paper is concerned was only observed on raising the pH. Evidence is, however, presented that the principal mechanism causing the pH drift is the enzymic demethylation of pectin. The question has been investigated in some detail because pH adjustment is an essential preliminary to most work on the liberation of viruses and normal proteins from leaves, and it is an advantage to know what changes the pH adjustment itself brings about.

MATERIAL AND TECHNIQUE

For most of the work leaves of *Nicotiana tabacum* var. White Burley were used, as they were available during the whole year from plants grown in the glasshouse. Other plants, listed below, were also used.

The leaves were minced with a domestic meat mincer, the sap squeezed out through madapollam, and the residue washed by suspending in about five times its own weight of water and squeezing out. After three washes the minced material was squeezed as dry as possible by hand and weighed portions taken. The material thus obtained is called *fibre* and contains about 25% dry matter. Fibre (10 g.) suspended in water (100 ml.) gives a mixture which can be stirred easily; vigorous stirring is necessary during the addition of alkali to prevent local raising of the pH much above 8. Chloroform was used as an antiseptic in fibre suspensions. For some of the work the minced fibre was ground finely in a triple-roller mill as described by Bawden & Pirie (1944). pH measurements were made with a glass electrode. Unless otherwise stated, experiments were carried out at room temperature. The analytical methods which were used are described in the last section (see Methods).

RESULTS

When minced fibre was suspended in water at its own pH (5·8–6·2) the pH remained constant within 0·4 unit for several days. The marked drift of pH towards the acid side took place only after raising of the pH by addition of alkali, and the pH never drifted to below the original value.

The amount of alkali needed to stabilize the pH at 8 varied with different batches of fibre, the average amount for minced fibre being 38 ml. 0·2N-KOH/100 g. wet wt. of fibre. Minced fibre of the leaves of *Nicotiana glutinosa*, wild beaked parsley (*Chaerophyllum sylvestre*), beech (*Fagus sylvatica*), groundsel (*Senecio vulgaris*) and dock (*Rumex obtusifolius*) showed the drift and took up a similar amount of KOH. At the beginning of neutralization larger amounts of alkali were required to raise the pH of the suspension to 8 than were necessary towards the end. As the fibre is neutralized it swells and its water-holding power increases. When fibre that has been finely ground in the triple-roller mill is washed by suspension in water and squeezing out, much of the leaf protein is extracted, and the residue has a lower nitrogen content than the original fibre. Washed milled fibre needs about twice as much alkali to stabilize its pH as an equal weight minced. About one-third of the total alkali taken up is

needed for the initial neutralization of milled fibre and the pH is stabilized more rapidly than with minced material. As the substance responsible for the drift was not extracted by washing the fibre, a cell-wall constituent was indicated.

Location of the acid formed

To determine whether the acid formed was extracted from the fibre, *potassium* was estimated both in the neutral extract obtained by squeezing out the fluid from the fibre after the pH had been stabilized, and in the fibre itself. The potassium content of the original fibre, and of fibre after neutralization, was determined by the method given later (see Methods).

The following result is typical: 12 g. minced fibre (dry wt. 2·78 g.) were neutralized by 12·5 ml. 0·091 N-KOH. The potassium content of the original fibre was 3·1 mg., of the neutral extract 19·2 mg. and of the neutralized fibre 24·5 mg. The total potassium accounted for is 40·6 mg., i.e. 92% of the amount added. 43% of the added potassium was in the neutral extract, indicating that the greater part of the acid produced remained on the fibre. The potassium is not simply adsorbed, for when 20 g. fibre at pH 6 were suspended in a potassium sulphate solution containing 100 mg. potassium, after 24 hr. 98·5 mg. were found still in solution.

To test for volatile acid a neutral extract from fibre which had needed 3·3 ml. 0·2N-KOH for neutralization was distilled after acidification with H_2SO_4. Only 0·05 ml. 0·2N-KOH was required to neutralize the acid in the distillate, indicating that there was a negligible amount of volatile acid present.

Carbon-dioxide production in the fibre suspension

That the soluble acid was carbonate, some of the carbon dioxide being formed by metabolic changes in the fibre and some being picked up from the air, seemed probable. Carbonate was determined in the neutral extract, on samples removed at intervals during neutralization, by measurement of the CO_2 liberated on acidification in the Van Slyke manometric gas apparatus (Van Slyke, Page & Kirk, 1933).

Minced fibre (12 g.) was suspended in 150 ml. boiled distilled water in a wide-mouthed bottle, having a stopper holding a burette containing 0·091 N-KOH, and a sampling tube. The latter was a glass tube of 1 cm. diameter reaching nearly to the bottom of the bottle, and having organdie tied over the lower end to prevent fibre being removed when samples were taken. Phenol red was used as an internal indicator in the suspension. Chloroform was omitted as it interfered in the CO_2 determinations.

The results are given in Fig. 1. At the end of the experiment carbonate accounted for 39% of the added alkali, which is in agreement with the value for soluble acid. Making allowance for the carbonate content of the KOH and the CO_2 picked up by a solution at pH 8 from air during sampling, the metabolic production of CO_2 would account for 25% of the added alkali.

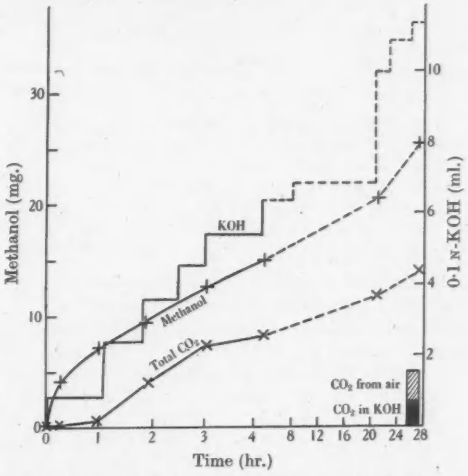

Fig. 1. Relationship between addition of KOH to minced fibre, carbonate content of the solution and methanol liberated. KOH added to 12 g. minced fibre in 150 ml. water to keep the pH at 8. Methanol and CO_2 determined at intervals. Curves are plotted so that ordinates for equimolecular quantities of KOH and methanol are the same, i.e. 1 ml. N-KOH = 32 mg. methanol. CO_2 is plotted in terms of its KOH equivalent. CO_2 from the air and KOH is shown in the bottom right-hand corner.

Titration curves made on a neutral extract showed that it had a low buffering power except in the region of pH 6·4 which is pK_1 for carbon dioxide. This confirmed the finding that the greater part of the acid remained on the fibre and that the soluble acid was carbonate.

Portions of minced and milled fibre were boiled at pH 6 and, when cool, alkali was added to neutralize the suspension. The amount of alkali needed was small and there was little pH drift. The total amount of 0·16N-KOH taken up by 12 g. boiled minced fibre was 1·4 ml., whereas it was 8·0 ml. for unboiled. An enzymic reaction was therefore indicated as the cause of the pH drift.

Liberation of methanol from fibre under various conditions

Ethanol appeared to be absent from a neutral extract of milled fibre, for a distillate from the extract did not give the iodoform reaction. Methanol, on the other hand, was present as shown by (1) forma-

tion of methyl salicylate on warming with sodium salicylate and sulphuric acid; (2) development of purple colour with Schiff's reagent on standing in the presence of conc. H_2SO_4 after oxidation with potassium permanganate. The presence of an appreciable amount of methanol having been established, quantitative determinations were made by a modification of a method given by Schryver & Wood (1920) (see Methods).

The liberation of methanol throughout neutralization was followed in both minced and milled fibre.

The fibre was suspended in water in a bottle with a burette and sampling tube through which samples for methanol determinations were removed at intervals. When the total methanol present at a particular time was calculated, allowance was made for the changes in volume due to removal of samples and the addition of KOH, and for the methanol content of the samples removed previously.

The results (Fig. 1) showed that there was an increase in methanol during neutralization.

Phosphate solution (0·1 M-Na_2HPO_4) adjusted to pH 8 had sufficient buffering power to hold the pH of a mixture containing 3·0 g. milled fibre and 60 ml. phosphate solution steady within 0·2 pH unit. Methanol was determined in such a mixture at intervals. Fig. 2 gives the results, which also show

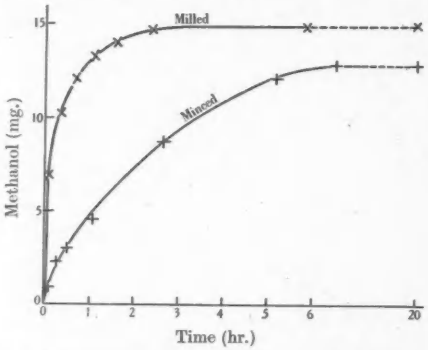

Fig. 2. Comparison of rate of liberation of methanol from minced and milled fibre. Portions (3 g.) of minced or milled fibre in 60 ml. phosphate buffer (0·1 M) at pH 8.

that with fibre in buffer solution the liberation of methanol is slower with minced than with milled fibre. Liberation of methanol from buffered fibre is more rapid than from fibre neutralized with alkali.

For the following experiments milled fibre only was used as it provided a more homogeneous substrate than minced fibre. A comparison was made of the rate of liberation of methanol from fibre at pH values from 6·0 to 8·0. The results given in Fig. 3 show that the liberation is most rapid at pH 8 and that there is some demethylation at pH 6, the natural pH of the fibre suspension.

The total methanol liberated from 3 g. fibre boiled at pH 6 in water, cooled, and then suspended in 60 ml. phosphate solution at pH 8 for 20 hr., was 1·74 mg. The distillate from 3 g. fibre boiled at pH 6 contained only 0·3 mg. methanol, showing that the

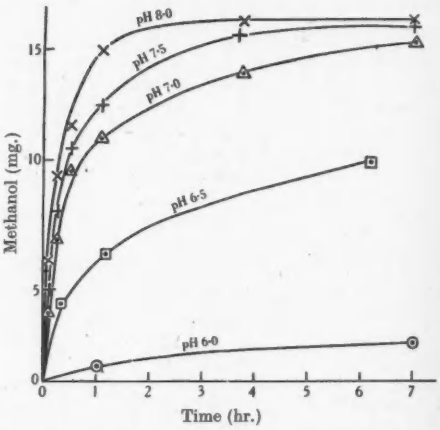

Fig. 3. Comparison of methanol liberation at different pH values. Portions (3 g.) of milled fibre in 60 ml. phosphate buffer solutions (0·1 M) at pH values from 6 to 8.

fibre had not been previously demethylated by boiling at pH 6, and therefore that the great decrease in the amount of methanol produced was due to the destruction of the demethylating enzyme.

A suspension of 3 g. milled fibre in 100 ml. water to which 1 ml. 5 N-NaOH had been added was boiled and 50 ml. distillate collected. The methanol content of the distillate was 14·4 mg., which corresponded with the amount produced enzymically at pH 8. von Fellenberg (1918) found that ester-combined pectin methyl groups were split off by dilute alkali, whereas the ether-linked lignin methyl groups remained attached.

Non-enzymic demethylation. Fibre (3 g.) that had been boiled at pH 6 was boiled in 100 ml. 0·05 N-NaOH; the methanol found in the distillate was 15·6 mg., showing that fibre in which enzyme had been destroyed could be demethylated by boiling with dilute alkali.

The non-enzymic demethylation of boiled fibre at different pH values at room temperature was investigated. Portions (0·5 g.) of boiled fibre were suspended in 10 ml. solution for 20 hr. and methanol determined. Table 1 shows that although there is some non-enzymic demethylation at pH 8 it is more extensive in solutions of higher pH.

Heat stability of enzyme. Portions of fibre were suspended in water and kept at different temperatures for 15 min. and cooled; each portion was then

Table 1. *The non-enzymic liberation of methanol from boiled fibre*

(0·50 g. boiled fibre in 10 ml. solution at different pH values for 20 hr. Room temperature.)

pH	Medium	Methanol (mg.)
6·05	0·1 M-phosphate solution	0
6·88	,,	0
7·95	,,	0·31
9·40	,,	0·75
12·3	0·02 N-NaOH	1·44
Boiled in 0·02 N-NaOH		2·34

squeezed out and suspended in 0·1 M-phosphate solution at pH 8 and methanol was determined at intervals. The results are given in Table 2.

Table 2. *Methanol liberation in heated fibre*

(Suspensions of 3 g. milled fibre in 50 ml. water, at pH 6, kept at the temperatures given in the first column for 15 min. before suspending in 50 ml. 0·1 M-phosphate at pH 8.)

	Methanol (mg.) liberated in		
°C.	2 hr.	17 hr.	41 hr.
18	12·3	13·5	13·8
60	7·3	11·5	12·0
70	6·1	10·7	11·2
80	4·7	9·6	10·0
90	1·8	3·1	3·5
100	1·3	2·7	2·7

Demethylation in the presence of sap

Neuberg & Kobel (1927) obtained pectase from the expressed sap of tobacco leaves which could demethylate extracted citrus pectin. Neuberg & Ottenstein (1928) showed that enzymic demethylation of pectin took place in a mince of tobacco leaves at the normal pH of the minced leaves. As demethylation is slow in well-washed minced or milled fibre at pH 6, an experiment was made with fibre in the presence of sap to obtain conditions comparable with those used by Neuberg & Ottenstein.

An examination was made of methanol liberation in fibre + sap under various conditions, and also from boiled fibre. The details of this experiment are given later (see Methods). The results are given in Table 3. This shows that demethylation of the fibre has taken place to the extent of 28 % at 16° and of 48 % at 40°, in 24 hr. at pH 5·9. At pH 8 in 24 hr. demethylation was practically complete and was more rapid at 40° than at 16°. There was little increase in the amount of methanol from boiled fibre at pH 5·9 and a small increase at pH 8 due to non-enzymic demethylation. These results are somewhat different from those of Neuberg & Ottenstein (1928), who found that 50 % of the methanol present in the form of esters was liberated in 2 hr. at pH 6.

Table 3. *The liberation of methanol from fibre + sap under various conditions*

(Values at pH 5·9 obtained by adding 40 ml. water to 10 g. fibre + sap. Values at pH 8·1 obtained by neutralizing the mince with alkali and then adding 40 ml. 0·1 M-Na_2HPO_4 solution at pH 8·1.)

	Methanol (mg.) liberated in				
Treatment	10 min.	30 min.	2·25 hr.	6 hr.	24 hr.
pH 5·9, 16°, boiled	—	1·37	—	1·51	1·70
pH 5·9, 16°	0·28	1·31	2·25	2·86	4·95
pH 5·9, 40°	—	2·32	4·13	6·76	8·43
pH 8·1, 16°, boiled	—	—	1·93	2·32	3·27
pH 8·1, 16°	—	10·00	13·56	16·05	15·94
pH 8·1, 40°	—	13·05	15·01	16·51	16·34
Boiled with NaOH ...		16·63			

The total amount of methanol liberated in the absence of sap

Minced fibre (10 g.) was washed with three 50 ml. portions of water and methanol determined in the washes. The fibre was then suspended in 100 ml. water and methanol determinations made during 5 days; in this time the pH fell from 6·34 to 5·63. The fibre was squeezed out and suspended in 80 ml. 0·1 M-phosphate solution at pH 8; methanol was again determined at intervals. Finally, the fibre was boiled with alkali and methanol determined in the distillate.

Table 4 shows that at pH 6 demethylation is at first fairly rapid, even in the absence of sap, but slows off considerably. Then at pH 8 the fibre is rapidly and completely demethylated.

Table 4. *The total liberation of methanol from minced fibre in the absence of sap*

(10 g. fibre were washed 3 times, then suspended in water for 5 days and afterwards in phosphate buffer (0·1 M) at pH 8 for 2 days. The suspension was finally boiled with alkali. Samples were taken at intervals, as shown, and methanol determined.)

Treatment		Methanol (mg.)
Three washes, 1 hr.		5·4
Soaking at own pH:	1 day	4·8
	2 days	6·2
	5 days	7·4
Soaking at pH 8:	2 hr.	12·8
	20 hr.	19·1
	2 days	20·5
Boiling with NaOH		0·4
	Total	33·7 mg.

Examination of various enzyme preparations for demethylating activity

Experiments were made to find means of demethylating boiled fibre enzymically. Some commercial enzyme preparations were tested for demethylating activity. Portions (2 g.) of boiled

fibre were suspended in 40 ml. 0·1M-phosphate solution at pH 8 with, respectively, 80 mg. trypsin (British Drug Houses Ltd.), 80 mg. taka-diastase (Parke Davis and Co.), 170 mg. 'Luizym' (Luitpold-Werk, Munich). Another 2 g. portion was tested with snails' stomach juice, the quantity used being equivalent to the juice from one stomach. None of these enzymes was effective in demethylating boiled fibre.

Boiled fibre (3 g.) was suspended in 88 ml. centrifuged sap of *Nicotiana tabacum* that had been adjusted to pH 8. The high buffering capacity of the sap was sufficient to hold the pH at the initial value. The methanol content of the sap was 4·26 mg., and after 48 hr. there was an increase of 11·43 mg. It is clear, as Neuberg & Kobel (1927) found, that the sap contains a demethylating enzyme. It might be thought that the methanol found in the sap was produced only on mincing, as the result of the enzyme and substrate being brought together. However, methanol was found in the juice squeezed out from frozen whole leaves, which suggests that the methanol was preformed.

Extraction of enzyme from fibre. Preliminary experiments showed that water extracts of milled fibre at pH 6 had no demethylating activity on boiled fibre, but that extracts at pH 8 made either with alkali or phosphate had strong activity. Extracts at pH 8 from bean and tomato fibre also could demethylate tobacco fibre. By distilling *in vacuo* below 30° the extracts could be concentrated and methanol removed. The active portion of the extracts could be precipitated by half-saturation with ammonium sulphate.

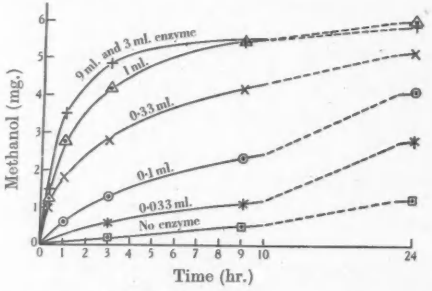

Fig. 4. Dependence of methanol liberation on enzyme concentration. Portions (2 g.) of boiled milled fibre with amounts of enzyme concentrate 9·0–0·033 ml. in phosphate buffer (0·1 M) at pH 8.

Milled tobacco fibre (15 g.) was demethylated in phosphate solution (pH 8) and the neutral extract concentrated to 50 ml. Portions (2 g.) of boiled fibre were taken and different amounts of the concentrated fibre extract, from 9·0 ml. down to 0·033 ml., were added and the volume made up to 40 ml. with 0·1M-phosphate solution pH 8. Boiled concentrated fibre extract (3 ml.) was added to one portion, and there was a control to which no enzyme was added. Samples were removed at intervals. The results given in Fig. 4 show that the amount of methanol liberated was dependent on the enzyme concentration. The curve for 9 ml. of enzyme solution was the same as that for 3 ml. except that the 20 min. value was slightly higher.

Demethylation of pectin. Enzyme concentrates were tested for their ability to demethylate pectin extracted from tobacco leaves by ammonium oxalate solution (see Methods). The ammonium oxalate-extracted fibre (3 g.), when boiled with 0·05N-NaOH, gave 2·1 mg. methanol, which showed that most of the methanol-producing substance had been removed. When neutralized fibre was extracted with ammonium oxalate solution the precipitated pectic material had no methoxyl content.

Pectin was dissolved in water; to one portion, phosphate buffer at pH 6 was added + 15 ml. enzyme solution (= amount of enzyme extracted from 1·5 g. fibre); to two other portions, phosphate buffer at pH 8 was added, and 15 ml. enzyme to one of them. Samples were removed at intervals and methanol estimations made. As soon as the sample was withdrawn acid was added to it to lower the pH to 4 in order to stop any further action and to prevent demethylation when the sample was distilled. On acidification of the samples from the preparations with enzyme a gelatinous precipitate was obtained.

The results given in Table 5 show that the extracted enzyme is effective in demethylating extracted tobacco pectin. There was some demethylation at pH 8 without enzyme. At pH 8 with enzyme demethylation is more rapid than at pH 6.

Table 5. *Liberation of methanol from extracted tobacco-leaf pectin*

(200 mg. portions of pectin in 40 ml. water + 25 ml. 0·1M-phosphate solution (at pH 6 or at pH 8) + 15 ml. enzyme solution.)

	Methanol (mg.) liberated in			
	5 min.	45 min.	3 hr.	17 hr.
pH 6 + enzyme	1·15	2·40	3·79	7·05
pH 8, no enzyme	0	Trace	—	1·57
pH 8 + enzyme	2·46	4·45	6·67	8·96

100% liberation = 9·9 mg.

Demethylation in minced leaves of members of the Cucurbitaceae

In the plants so far considered the pH of the expressed sap was 6 or less. It was thought that an investigation of the methanol liberation in plants with more alkaline sap might be of interest. Bawden & Pirie (1937) recorded the pH of the expressed sap of cucumber leaves as between 7 and 8. Cucumber leaves from glasshouse plants gave on mincing a

sap with a pH of 7·2. After three washes the pH of the fibre suspension (prepared as previously described) had risen to 9·6. Other members of the Cucurbitaceae were also found to have relatively alkaline saps: white bryony (*Bryonia dioica*) pH 6·6–7·2, marrow (*Cucurbita pepo*) 6·9–7·4 and melon (*Cucumis melo*) 7·4. On washing the fibre of these leaves the pH increased; the value of pH 9·4–9·6 was reached, which was maintained but not exceeded when washing was continued. In these plants the methanol content of the sap was from 0·25 to 0·5 mg./ml., i.e. from five to ten times as high as in tobacco. The freeze-juice from whole leaves had also a higher methanol content. The addition of alkali to bring about demethylation in bryony, etc., is not necessary; methanol is liberated spontaneously owing to the high pH. When the fibre of these plants is boiled, demethylation takes place rapidly although the enzyme is destroyed. A concentrate of the extract of bryony fibre soaked at the pH reached on washing, i.e. 9·4, was effective in demethylating boiled tobacco fibre. The phenomenon of rise of pH on washing is at present under investigation.

DISCUSSION

All the observations make it clear that the pH drift is mainly due to the enzymic breakdown of pectin to methanol and pectic acid.

The slower liberation of methanol from fibre neutralized with alkali than from fibre in buffer solution is due to the pH falling between each addition to a value where demethylation is slower. Minced fibre contains a large proportion of intact cells, whereas in milled fibre all the cells have been ruptured and torn apart. There is therefore a larger surface area of pectin exposed to the enzyme and the solution of higher pH can permeate more easily. Because of this, demethylation in milled fibre is more rapid than in minced.

Methanol liberation is considerably affected by keeping fibre at different temperatures before neutralization (Table 2). The values in Table 2 and Fig. 4 show that the relationship between enzyme and substrate, in the form in which it is present in boiled fibre, is complex, and that with small amounts of enzyme the rate of liberation of methanol is such that the total possible methanol would not be produced.

Neuberg & Kobel (1927) gave the pH optimum for the action of tobacco pectase on extracted citrus pectin as 5·6, while Kertesz (1936, 1937) stated that pectin-methoxylase (pectase) had no pH optimum, the action increasing with decreasing hydrogen-ion concentration until the effect of the alkalinity of the medium superseded the enzymic action. In the present investigation, although the enzymic demethylation of extracted pectin was fairly rapid at pH 6, it was very slow at this pH when the pectin was on the fibre. As the pH was raised the action of the enzyme increased, but even at pH 8 the non-enzymic demethylation was appreciable. Owens, McCready & Maclay (1944) gave pH 9 as the maximum value for the *in situ* enzymic demethylation of citrus-peel pectin; at higher pH values the alkaline de-esterification was considerable.

The presence of pectase in the leaf-sap of many plants is well known, but it is of interest that a pectin-demethylating enzyme should remain on the fibre and only be extracted when the pH is raised.

METHODS

Potassium determinations. The potassium was precipitated as potassium-silver-cobaltinitrite with 25% w/v Analar sodium cobaltinitrite in the presence of $AgNO_3$. The precipitate was washed with aqueous acetone, acetone and finally ether and allowed to dry. It was then dissolved in dilute HNO_3 and ethanolic ammonium thiocyanate added. This gave a blue colour (Breh & Gaebler, 1930), which was compared in a visual colorimeter with standards from a range containing 0·1–0·5 mg. of potassium. Weighed amounts of fibre which had been dried at 100° were ashed in a porcelain crucible with H_2SO_4 at 500°. The hot-water extract of the ash was filtered and potassium determined in the filtrate. To the neutral extract a few drops of glacial acetic acid were added and the solution filtered to remove precipitated protein. A sample was taken containing less than 0·5 mg. potassium.

Methanol determinations. A method for estimating methanol described by Schryver & Wood (1920) was based on the colour reaction between phenylhydrazine hydrochloride, potassium ferricyanide, hydrochloric acid and formaldehyde. The methanol was converted to formaldehyde by ammonium persulphate. This method is laborious and not suitable for routine estimations, as it is necessary to determine the amount of persulphate required for the oxidation of each sample. Snell & Snell (1937) suggest that the potassium permanganate and oxalic acid reagents used in the modified Denigès method for methanol estimation could be used instead of ammonium persulphate. Solutions of $KMnO_4$ (3 g.) in 85 ml. water + 15 ml. 85% (w/w) H_3PO_4 and oxalic acid 5% (w/v) in 49% (w/w) H_2SO_4 were tested. The presence of conc. H_2SO_4 reduced the sensitivity of the test so much that it was useless. With lower concentrations of H_2SO_4 the permanganate solution did not clear. However, when a more concentrated solution of oxalic acid was used, and the solution warmed, it became clear and colourless and the H_2SO_4 could be omitted.

The estimations were made as follows: Samples of methanol-containing solutions (0·5–5·0 ml.) with a methanol content of 0·03–0·40 mg. were distilled in the steam distillation apparatus described by Markham (1942). Most of the samples in which methanol was to be determined were coloured and cloudy so that it was necessary to use a distillate. Possible interfering substances were also avoided by this means. To ensure complete recovery of methanol, 5 ml. of distillate were collected. To the distillate 1 ml. $KMnO_4$ (as above) was added, and after standing 10 min. 1 ml. 10% (w/v) aqueous oxalic acid. The time of oxidation is of importance because although on longer standing the

amount of methanol converted to formaldehyde is increased, there is also a great increase in the amount further oxidized to formic acid. Under the conditions used about 26% of the methanol was converted to formaldehyde. After the addition of oxalic acid the solution was warmed. When cool 2 ml. 1% (w/v) phenylhydrazine hydrochloride was added followed by 1 ml. 2% (w/v) potassium ferricyanide and 3 ml. conc. HCl, making a total volume of 13 ml. On the addition of the ferricyanide solution a precipitate formed which disappeared on the addition of the HCl, when the cherry-red colour developed immediately. Freshly prepared reagents must be used or the precipitate does not disappear and the colour developed is paler. The intensity of colour was compared in a visual colorimeter with standards containing 0·04–0·32 mg. methanol prepared from a solution containing 0·08 mg./ml. methanol, in the same way and at the same time. The colour is fairly stable but fades on long standing, so comparisons were made within 15 min. of the colour being developed.

In all experiments when serial estimations of methanol were made samples were kept until there was a convenient number (about 12) to be assayed in a batch. Samples free from suspended fibre gave constant values for methanol, whether assayed immediately or after being kept 24 hr.

Experiment to determine the rate of demethylation in the presence of sap. Leaves were minced and 10 g. portions of fibre + sap (dry wt. 1·15 g.) weighed out as quickly as possible. Three portions were each diluted with 50 ml. water, the pH on dilution being 5·91. One of these was kept at room temperature (16°), another at 40° and the third boiled and then kept at 16°. Two portions were adjusted to pH 8 with 0·2N-NaOH and diluted with 50 ml. 0·1M-phosphate solution (pH 8·1). One of these portions was kept at 16° and the other at 40°. Another portion was diluted with 10 ml. water, boiled, and when cool adjusted to pH 8 and 40 ml. 0·1M-phosphate solution added. A further portion was distilled with 1 ml. 5N-NaOH added to the suspension. The methanol content was determined in the distillate to give the value for the total amount of loosely combined methanol. The first sample was taken 10 min. after mincing, and the methanol concentration found was taken as the initial value for the series.

Extraction of pectin. Well-washed milled fibre was extracted with ethanol to remove pigments, then suspended in water and boiled for 5 min. to destroy the demethylating enzyme. Aqueous ammonium oxalate (0·5% w/v) was used for pectin extraction. This was carried out at pH 4 and 25° for 48 hr., the extraction solution being changed frequently. An equal volume of 95% ethanol was added to the extract in order to precipitate the pectin. The precipitate was dissolved in water and reprecipitated three times. After drying over H_2SO_4 the product had a methoxyl content of 4·8%. When dissolved in water the solution was opalescent, rather viscous, and had a pH of 5·4. The methoxyl content of the pectin was low but it was possible to use the material to demonstrate the demethylating action of the enzyme on pectin.

SUMMARY

1. The drift of pH towards the acid side observed when minced tobacco leaves are neutralized with alkali is mainly due to the enzymic demethylation of pectin when the pH is raised above that of the normal sap. The metabolic production of carbon dioxide is also in part responsible for the drift.

2. The liberation of methanol is more rapid in milled than in minced leaf fibre, and at pH 8 more rapid than at pH 6, whether in the presence or absence of sap.

3. Heating to 100° prevents the pH drift, and the small amount of methanol liberated at pH 8 is due to non-enzymic demethylation.

4. The pectase is extracted from fibre at pH 8 but not at pH 6. The pH 8 extract will demethylate boiled fibre and extracted pectin.

5. Minced leaves of some Cucurbitaceae have a spontaneous alkaline drift when washed, which permits demethylation without the addition of alkali.

6. An improved method for the estimation of methanol is described.

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The Purification and Crystallization of Rennin

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The crystallization of urease by Sumner (1926) has been followed in the past few years by successful attempts to bring other enzymes into the crystalline condition. There has been some tendency to regard the crystalline form as an indication of a high degree of purity in the preparations concerned, but, as shown by Pirie (1940), this is theoretically unsound. Langmuir (1938) has pointed out that colloids may form coacervates having regular molecular orientation in one, two, or three dimensions. He regards some protein crystals as solid tactoids belonging to one of these three classes and holding a position intermediate between solutions and true crystals. Tactoids will thus tend to be purer than the solutions from which they are formed, but the purifying effect of precipitation will be less than in the formation of true crystals. Nevertheless, though crystalline form is not of itself complete and final evidence of either purity or true crystallinity, it is a matter of experience that unpurified enzymes cannot be crystallized and that quite small quantities of some impurities prevent crystallization (e.g. 1% of mucin prevents the crystallization of pepsin) (Northrop, 1939). It is therefore safe to regard an enzyme preparation which can be crystallized as likely to be purer than one which cannot.

The crystallization of rennin has previously been attempted by Tauber & Kleiner (1932) and by Hankinson (1943). The former obtained a small quantity of minute spheres by a method which they consider impracticable. The latter claims greater success in the crystallization and shows a photograph. Hankinson states that photography was difficult owing to Brownian movement of the particles, and to their gradual disappearance when placed on the microscope slide.

The present communication describes, after summarizing the experience gained in the preliminary experiments, the preparation and some properties of the rennin crystals shown in Pl. 1, Figs. 1, 2.

METHODS

(1) *Measurement of activity.* Throughout the preparations the activities of the various fractions were determined by measuring the time required for 1 ml. of the diluted enzyme to clot 10 ml. of standard substrate at 30 (±0·1)°. The substrate was made by dissolving 12 g. of good-quality spray-dried skim milk powder in 100 ml. of 0·02 N-$CaCl_2$. All the minor details of the technique of measurement, e.g. time of keeping milk at 30° before adding rennin, method of determining end-point, etc., were rigidly standardized. The end-point was observed by allowing a thin film of the milk to flow from a glass rod down the side of the test-tube containing the milk. When clotting occurred, the almost invisible film broke into a number of white particles. Reproducible results were obtainable as long as clotting times were kept within 4–6 min. The activity of the solution was then obtained from the formula

$$\text{Rennin activity} = \frac{100d}{t} \text{ Rennin units (R.u.)/ml.},$$

where d = dilution of the rennin before adding to the milk, and t = clotting time in sec.

(2) *Determination of purity.* Towards the end of the first preparation, figures for the comparative purity of the fractions were obtained by determining the activity per unit of nitrogen, but later, activities per unit of organic dry weight were used since they indicated also comparative freedom from non-nitrogenous impurities.

The dry-weight measurements were made by weighing on the microbalance the residue remaining after 16–20 hr. drying at 98° of 0·1 ml. of the rennin solution, containing NaCl as the only added inorganic material, in a small weighed platinum dish. The solutions were subjected to a preliminary evaporation on a steam-heated platinum plate before placing in the oven at 98°. The NaCl content was determined on the same sample by careful ashing with a drop of concentrated H_2SO_4 and calculating back to chloride. It was easy to obtain the enzyme free from other electrolytes when it was in the crystalline or granular state merely by washing the filtered solid with saturated NaCl solution. Five rapid washings at the pump sufficed for the complete removal of sulphate (as judged by the addition of $BaCl_2$ to a solution of the washed precipitate) from a preparation crystallized in half-saturated $MgSO_4$. Although only empirical, since the precautions mentioned by Chibnall, Rees & Williams (1943) concerning the drying of proteins were not carried out, the figures obtained were reproducible, usually to within ±1%, and were thus of sufficient accuracy for following the preparation. The purity of the fraction could then be expressed as the number of units of rennin activity per mg. of organic dry weight.

(3) *Method of obtaining granular precipitates.* Granular precipitates could be obtained before the rennin was pure enough to crystallize by adding the salting-out agent sufficiently slowly. Saturated solutions of salts were used, the rate of addition being controlled by displacement by a weight descending into the salt solution from a shaft connected to the winding spindle of an ordinary clock. The rate of displacement could be varied by varying either the diameter of the weight or that of the rotating shaft.

(4) *Proteolytic activity.* This was determined by a method similar to that of Anson (1938), a photoelectric absorptiometer being used for the colour readings. The pH values of the haemoglobin solutions were adjusted by adding previously determined quantities of HCl, and finally measured with the glass electrode after allowing 1 or 2 days at 2° for the establishment of equilibrium.

PREPARATIONS

Small scale (preliminary). A crude rennin solution was obtained by extracting eight dried salted calf abomasa with 10% NaCl for about 20 hr. with continuous stirring. The rennin from just over 3 l. of the extract was adsorbed on alumina precipitated *in situ* by the addition of a solution of 60 g. of potash alum followed by enough NaOH solution to bring the pH back to 6. After the alumina complex had been filtered and washed with water, the rennin was eluted with 1 l. of 0·2 M-sodium phosphate, pH 6·6. This process gave a considerable increase in purity without the necessity of preparing the special alumina usually employed for the adsorption of enzymes. [See, for example, Lüers & Bader (1927).] (It is possible to elute some rennin with 5–10% NaCl at a pH of 6·8. This appears to give a greater increase in purity, but the losses due to uneluted rennin may be very high.) The eluate was precipitated by saturating with NaCl, redissolved in 240 ml. of distilled water, and again salted out. A repetition of the adsorption and elution procedure and another salting out finally gave 10 ml. of a suspension with a total activity equivalent to slightly less than that of a normal dried abomasum. Alkali was added to this suspension until solution occurred (pH below 7), followed by sufficient acid to produce a faint turbidity. On warming, this turbidity increased, and therefore the suspension was cooled to about −5°, when a clear solution was obtained; as the temperature rose slowly an active slightly granular precipitate was formed. A little more acid was added and the process repeated twice. A fourth precipitate, obtained in the same way but with gentle agitation during the warming period, consisted of larger granules. In all, granular precipitates with an activity amounting to about a thirtieth of that of a dried abomasum were obtained.

The above process was repeated, except for minor differences, on approximately four times the previous quantities, until the 'crystallizing' stage was reached, when precipitation by temperature change was replaced by salting out through a rotating semi-permeable membrane as suggested by McMeekin (1939). This resulted in a granular precipitate containing a proportion of spheroids. Recrystallization by the slow addition of $MgSO_4$ (saturated solution), gave a highly active precipitate consisting almost entirely of spheroids. Northrop (1939) has pointed out that enzymes are precipitated as spheres either when they are not quite pure enough to crystallize properly, or when the conditions are not suitable for crystallization. The above results therefore justified further experimentation.

Large scale. The first of these has been briefly reported (Berridge, 1943). Commercial rennet equivalent to about 80 l. of the usual cheese-making rennet was used as the starting material. The early part of the preparation was similar to that already described. The later part consisted in fractional precipitation by the slow addition of saturated magnesium or ammonium sulphate solutions, repeating the fractionations, and recombining the various fractions according to the usual fractional crystallization technique. The fate of the rennin at each stage of the process was followed at first by activity and nitrogen determinations, later by activity and organic dry-weight determinations. Finally, microscopic plates of crystalline appearance were obtained. It is unnecessary to describe this preparation in detail since the final one was simpler and more successful. During the first preparation, however, a number of interesting observations were made. (1) At the commencement of fractionation the least soluble, i.e. most easily salted-out fraction, was of low activity, and dissolved in very little water to give an extremely viscous solution. This was not examined but was presumably mucin. Since the mixture from which it was obtained was already considerably purified, it is possible that the preparations of earlier workers have contained this substance, which, if it be mucin, might account both for the high sulphur content reported [e.g. 1·19% by Tauber & Kleiner (1932) and 1·46% by Hankinson (1943)] and for the difficulty in crystallization. (2) Towards the end of the preparation only a comparatively slight further increase in purity could be effected by fractional adsorption on to alumina precipitated *in situ*, followed by complete elution with phosphate buffer. Thus the activity of the rennin adsorbed by 0·1% of alumina from a solution in about 400 ml., eluted and precipitated, was 130 units/mg. dry weight (R.u./mg.). That adsorbed by 1·0% of alumina from the filtrate, eluted and precipitated, had a purity of 141 R.u./mg., while that remaining in solution was 142 R.u./mg. (3) Treatment with commercial adsorption and decolorizing carbons* was without effect on crystallizability. (4) Adjusting a solution of partly purified rennet to pH 3·2 caused precipitation and a loss of 30% of the total activity in a day. The crystallizability of the remainder was not improved by this treatment. Fractionation by acid precipitation was of no value at this stage. (5) There was some indication that previous precipitation in the amorphous condition favoured subsequent crystalliza-

* I am indebted to Sutcliffe Speakman and Co. Ltd. for kindly supplying samples of carbons.

tion. (6) Crystallization by temperature change was always less successful than that by the slow addition of saturated salt solutions. (7) Successful crystallization was dependent on a number of factors not completely understood. For example, on dissolving rennin consisting of approximately 50% crystalline plates and 50% spheres (prepared by the slow addition of saturated $MgSO_4$ to a rennin solution), and precipitating by salting out through a rotating membrane, only spheroids were obtained. Reversion to the previous method gave about 3% plates. The more concentrated the rennin solution the smaller the proportion of crystals. The most important factor subsequently discovered was the very low solubility of the crystals. The solutions used at this stage were highly supersaturated.

A second large-scale preparation, in which wasteful adsorption processes were not used, and in which an attempt was made to keep the less pure fractions as small as possible, gave no improvement in yield and little in crystallinity. At the later stages destruction occurred as rapidly as purification so that increase in purity could only be effected by discarding large quantities of material. Even when this was done the requisite conditions for crystallization could not be found—the results of the previous preparation could not be repeated. Finally, a very few apparently perfectly crystalline rectangular plates, large enough to be visible to the naked eye, were obtained by filtering a saturated rennin solution into a vessel seeded with a very little solid $MgSO_4$ and rennin spheroids. There was too little of these crystals to be of any use, and after a few days the sample set aside for photography dissolved and reprecipitated as spheroids, while the sample used to seed a crystallization became covered with spheroids. Attempts to copy the conditions of filtration into a seeded vessel on a larger scale gave an increase in quantity and size of crystals, but the latter were very poor in quality, containing many rounded angles, and were mixed with numerous spheroids.

It seems probable that the conditions for crystallization depend partly on the impurities present, and that although this preparation was comparatively pure only the peculiar conditions described sufficed to prevent co-precipitation of the rennin and impurities.

Final preparation

In contrast to the earlier preparations this one was carried out with coarser fractionation so that larger impure fractions were discarded. The material was divided into four portions according to solubility in NaCl, the two middle portions combined, impurities adsorbed on to alumina, and the remaining rennin crystallized.

The preparation is summarized in the accompanying scheme (p. 182), and a detailed description is given below. Thymol, used in conjunction with the high salt concentrations and low temperatures, proved an effective preservative.

Precipitated commercial rennet* (equivalent to about 100 gal. of ordinary cheese-making rennet), specially prepared from fresh abomasa, was the starting material. Half of it was dissolved in ~40 l. of tap water, giving a solution of 540 R.u./ml. The solution was saturated with NaCl; the resulting flocculent precipitate was easily filtered off. The filtrate, containing 17 R.u./ml., was discarded. When redissolved in 27·5 l. the precipitate gave a solution of only 666 R.u./ml. Thus, of the original $21{\cdot}6 \times 10^6$ units only $18{\cdot}5 \times 10^6$ remained. NaCl was added to this solution until the filtrate from a small portion showed only about half the activity of the original, i.e. ~330 R.u./ml. After filtration, the second half of the rennin was precipitated by raising the NaCl concentration to saturation; the filtrate containing 30 R.u./ml. was discarded. Thus, that portion of the original material insoluble in saturated NaCl was divided into a more soluble and a less soluble portion. The rest of the raw material was similarly divided, but the division was carried out first, and each portion then precipitated with NaCl. The result was the same by this method, but the difficulties of filtration were very great. The first precipitate could be filtered off only by making the whole of the mixture into paper pulp and then extracting the filter cake with water to recover the rennin, whilst the second precipitate was removed with the aid of about 2% (w/v) aluminium phosphate formed *in situ*, and extraction of the cake with water as before. The scheme below regards the two processes as identical and the former process as being applied to the whole material.

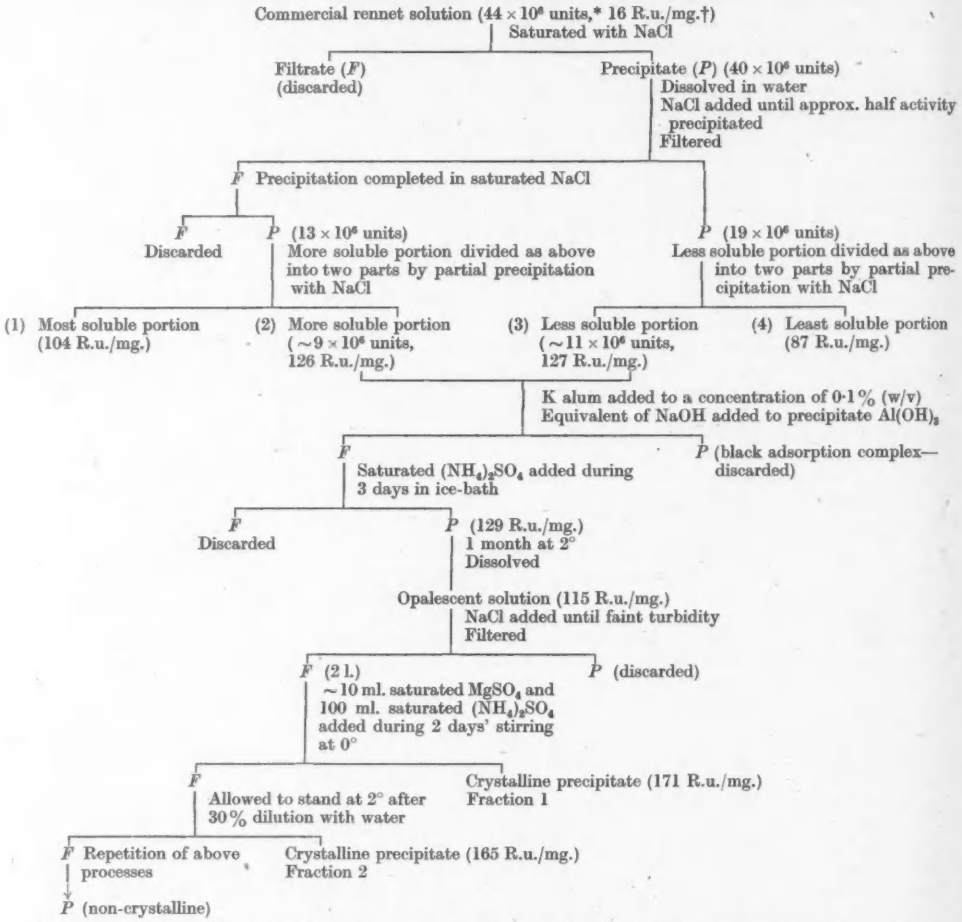
The united less soluble precipitates were dissolved in 14 l. and divided into a more and a less soluble portion by fractional precipitation with NaCl as already described. The first precipitate was flocculent and readily filterable. The second precipitate, which was formed slowly by adding ~100 g. of NaCl every 15 min. with continuous stirring until saturation, was sufficiently granular to be filtered easily on one paper. Thus the first less soluble portion was again divided into two portions according to solubility.

The second portion, consisting of the united more soluble precipitates, was similarly further subdivided into two portions. Here the first of these was granular and was readily collected on one filter. The attempted precipitation of the second resulted in an almost colloidal suspension which could not be filtered. Various methods of treating it were tried, such as re-solution and adsorption on alumina, but it was so unstable that by the time it had passed through these processes much of the activity had been lost. The remainder of this fraction, except the small amount of precipitate which had with difficulty been filtered off, was therefore discarded.

Thus, four precipitates differing in solubility and purity were obtained. The first contained most of the less soluble mucilaginous substances, the last,

* Kindly supplied by Benger's Ltd.

Preparation of crystalline rennin


Commercial rennet solution (44×10^6 units,* 16 R.u./mg.†)
Saturated with NaCl
Filtrate (F) (discarded)
Precipitate (P) (40×10^6 units)
Dissolved in water
NaCl added until approx. half activity precipitated
Filtered
F Precipitation completed in saturated NaCl
F Discarded
P (13×10^6 units) More soluble portion divided as above into two parts by partial precipitation with NaCl
P (19×10^6 units) Less soluble portion divided as above into two parts by partial precipitation with NaCl
(1) Most soluble portion (104 R.u./mg.)
(2) More soluble portion ($\sim 9 \times 10^6$ units, 126 R.u./mg.)
(3) Less soluble portion ($\sim 11 \times 10^6$ units, 127 R.u./mg.)
(4) Least soluble portion (87 R.u./mg.)
K alum added to a concentration of 0·1% (w/v)
Equivalent of NaOH added to precipitate $Al(OH)_3$
F
P (black adsorption complex—discarded)
Saturated $(NH_4)_2SO_4$ added during 3 days in ice-bath
F Discarded
P (129 R.u./mg.) 1 month at 2° Dissolved
Opalescent solution (115 R.u./mg.)
NaCl added until faint turbidity
Filtered
F (2 l.) ~10 ml. saturated $MgSO_4$ and 100 ml. saturated $(NH_4)_2SO_4$ added during 2 days' stirring at 0°
P (discarded)
F Allowed to stand at 2° after 30% dilution with water
Crystalline precipitate (171 R.u./mg.) Fraction 1
F Repetition of above processes
Crystalline precipitate (165 R.u./mg.) Fraction 2
P (non-crystalline)

* 1 unit clots 10 ml. milk in 100 sec. at 30°. † Rennin units (R.u.)/mg. = no. of units/mg. organic dry matter.

most of the more soluble proteins and peptones which had been adsorbed on the rennin last precipitated; the middle two contained least of both types of impurity. Activity and dry wt. of organic matter determinations were carried out on each of these fractions by the methods described, so that their purities could be expressed as the number of R.u./mg. of dry organic material present. The values obtained were 87, 127, 126 and 104 R.u./mg. respectively, in order of increasing solubility. The second and third fractions, containing 9×10^6 and 11×10^6 R.u. respectively out of the original 44×10^6 R.u., are thus considerably purer than the original material, which was about 16 R.u./mg.

These two precipitates were therefore combined. They dissolved readily when a little water was added. The resulting viscous solution set to a firm gel when left overnight at 2°. More water was therefore added and a permanent solution was obtained at a volume of ~3·5 l. All subsequent operations were carried out as far as possible in a refrigerator, or ice-bath. The very dark, turbid solution was clarified by adding 3·5 g. of potash alum crystals (in solution), followed by its equivalent of caustic soda solution (22·1 ml. N-NaOH), and filtering off the almost black adsorption complex. Although three large filters were used the filtration required nearly 4 days. About four-fifths

of the activity of the filtrate was precipitated by the drop-wise addition of 1·5 l. of saturated $(NH_4)_2SO_4$ during 3 days. The precipitate, in the form of microscopic masses of spheres, had a purity of 129 R.u./mg. which decreased, during storage for about a month at 2°, to 115 R.u./mg. The precipitate then dissolved only very slowly in 1 l. of water to give a turbid solution. This was purified by the addition of sufficient NaCl to cause a slight precipitate, and filtration. Overnight a small quantity of saturated $MgSO_4$ was added very slowly, through a capillary, to the stirred solution in an ice-bath. A precipitate of microscopic, rectangular prisms crystallized out. A further 100 ml. of saturated $(NH_4)_2SO_4$ were slowly added during the day and stirring continued overnight. An attempt was then made to separate the larger crystals by a short centrifuging. This necessitated a 30 % dilution with water to prevent the precipitation of amorphous rennin as the temperature of the solution rose. The precipitate was washed at the centrifuge with (*a*) half-saturated NaCl, (*b*) three-quarters-saturated NaCl, and finally suspended in saturated NaCl. A portion of this suspension was filtered at the pump and resuspended in distilled water to give a mixture containing per ml. 11·6 mg. dry organic matter, 5·8 mg. NaCl, and an activity of 1980 R.u., giving for the organic matter a purity of 171 R.u./mg. Thus the crystals were certainly rennin. The appearance of the suspension in saturated NaCl is shown in Pl. 1, fig. 2. The cracks are due to damage while centrifuging and washing. Thus the first crop, consisting of approximately 10 g. moist weight of rennin crystals, was obtained.

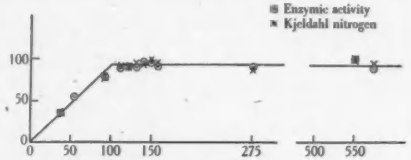
In spite of the addition of water, a second and larger crop of crystals separated from the mother liquor of the first crop during 3 days' standing in the refrigerator. These were photographed before washing (Pl. 1, fig. 1). They were washed as before, but at the pump, and stored in saturated NaCl with thymol added. Their purity was 165 R.u./mg.

By further slow additions of saturated $(NH_4)_2SO_4$ two more precipitates were obtained. They were granular but amorphous. Their purities were 119 and 112 R.u./mg.

PURITY OF CRYSTALLINE RENNIN

As a measure of the purity of their preparations earlier workers have used the activity per unit of dry weight or per unit of N content, and have been able to report large increases in these ratios, and thus in purity. Without an absolute measure of purity, however, these results are of limited value. The high activities reported are difficult to interpret and to compare, as differences of pH, calcium content and temperature, in addition to the natural variations in protein make-up of the milk, cause wide variations in susceptibility to rennet. It is thus very desirable to carry out absolute measurements of purity. Of these, solubility determinations in the presence of increasing quantities of solid phase give the best single criterion of purity.

Only one solubility experiment has, so far, been concluded. The graph shown in Text-fig. 1 was obtained. While far from perfect it indicates that the crystals are probably almost pure. It seems fairly safe to assume that the deviations from the ideal are due to experimental errors, except the slope of the first portion of this graph, which is < 1, probably owing to the presence of a small quantity of an impurity of very low solubility which prevents complete solution of the rennin, as suggested for pepsin by Steinhardt (1938). Indeed, a little insoluble residue was observed even in the most dilute sample. The extremely low solubility of the crystals indicated the need for a strongly salting-in electrolyte in the solvent. $MgCl_2$ was chosen; 50 g. of $MgCl_2.6H_2O$ and 15 g. of $CH_3COONa.3H_2O$ were dissolved in 1 l. and the solution adjusted to pH 5·4 with 10N-H_2SO_4. Crystals of crop 1 were recrystal-

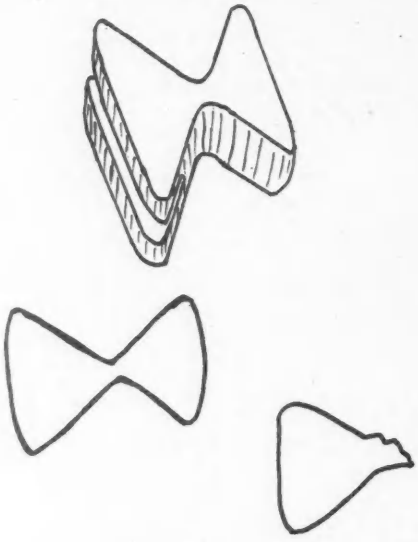


Text-fig. 1. Solubility of crystalline rennin with increasing quantities of solid phase. Solvent: 5 g. $MgCl_2.6H_2O$, 3 g. sodium acetate ($CH_3COONa.3H_2O$)/100 ml.; pH, 5·4. Ordinate: Activity of supernatant (R.u./ml.) = nitrogen of supernatant (mg./ml. × 1170).

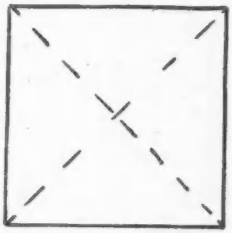
lized twice from this solvent (as described below, in detail, under 'Properties'), washed five times with small portions of solvent, allowing several minutes in each change of solvent, and resuspended to give a mixture containing 5500 R.u./ml. Dilutions of this were equilibrated with solvent in 6 ml. test-tubes in a container rotated in a vertical plane, an air bubble in each tube acting as stirrer. No traces of denaturation at the solvent-air interface were noticed. The container of tubes was immersed in a bath at 1–2°. After 5 days' equilibration, each tube was centrifuged for 7 min. in ice (packed in a 50 ml. metal cup of the International Centrifuge supported in cotton-wool in a 250 ml. metal cup), and the supernatant solution analyzed for activity and total nitrogen. Special care was necessary when removing the bung to avoid contaminating the solution with crystals collected on the side of the tube above the level of the liquid.

PROPERTIES OF CRYSTALLINE RENNIN

(*a*) *Solubility.* While crystalline proteins are generally less soluble than their amorphous forms the difference with rennin is very striking. Amorphous rennin precipitated by salting-out, whether finely divided or in the form of spheroids, cannot be



2*a*.



2*b*.

Text-fig. 2. *a*, crystal fragments remaining after dissolving most of the rennin. *b*, structure of some original crystals showing diagonal markings (faint) related to shape of fragments shown in *a*.

washed with distilled water. As soon as the excess of salt has been removed, the rennin dissolves in the dilute saline remaining, to form a gummy mass; it is apparently miscible with dilute salt solutions in almost all proportions. The crystals, however, may be washed with water, and however slow the washing, the loss is quite low. Even in the strongly salting-in solution used for the solubility experiment the solubility is very low, being approximately 0·06 g./100 ml. The solubility in distilled water has not yet been determined. If it is correspondingly lower, rennin may, like lactoglobulin, be another useful protein for solubility work in general, since it will not appreciably affect the dielectric constant of its own solution.

The low solubility of the crystalline form may account in some degree for the difficulty first experienced in obtaining crystals. The solutions used in the early preparations were so highly supersaturated that they could not be expected to crystallize readily.

The experiment already described has shown that the solubility of the crystals is independent of the amount of saturating body, but that a small proportion of the rennin did not dissolve even in the most dilute sample. The residue remaining after most of the rennin had dissolved contained a few fragments of characteristic shape (shown in Text-fig. 2*a*), apparently related to the structure of the crystals as indicated in Text-fig. 2*b*. The diagonal lines occur on only a few of the original crystals and are too faint to photograph in the ordinary way; they can be seen by only very careful examination. They may indicate planes in the crystal where the impurity of low solubility, believed to exist, has been deposited and has later prevented solution of the rennin.

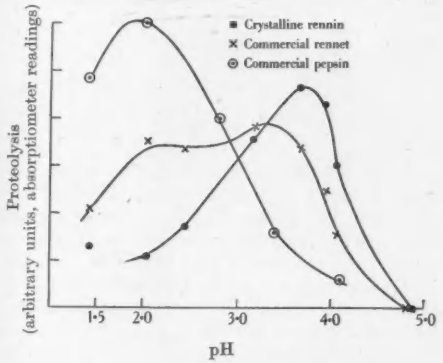
(*b*) *Recrystallization.* The difficulty of dissolving the crystals has already been described. This was partially overcome by raising the pH of the mixture to 6·8. In this way, 10 g. of filter cake of crystals moist with saturated NaCl could be dissolved in 800 ml. of approximately 0·06 M-NaH_2PO_4. After solution and filtration, the pH was adjusted to between 5·4 and 5·5. The very slow addition of saturated $(NH_4)_2SO_4$ to such a solution, followed by storage in the refrigerator as soon as crystals began to appear, led to the formation of a thick layer of crystals on the bottom of the vessel. Subsequent attempts to crystallize from this type of solution by the continual very slow addition of saturated $(NH_4)_2SO_4$, with continuous stirring, led to the precipitation of spheroids, in the centre of which the cubic crystals used for seeding could be seen. Precipitates of this type dissolved very rapidly in the $MgCl_2$-sodium acetate buffer used for the solubility experiment, to give a solution from which good crystals began to be formed almost at once. The recrystallization procedure finally adopted was, therefore: solution at pH 6·8, precipitation in the amorphous condition with $MgSO_4$ or $(NH_4)_2SO_4$ at pH 5·4, and solution in $MgCl_2$-sodium acetate buffer at pH 5·4, from which crystallization readily occurred.

(*c*) *Effect of desiccation.* A small quantity of filtered crystals was kept for several weeks at room

temperature in a desiccator over P_2O_5, without loss of crystalline form. The activity was difficult to measure owing to the extreme slowness of solution even in a large excess of distilled water. A suspension of 3·5 mg. of crystals in 1 l. of distilled water reached its maximum activity after 4 hr. although still not completely dissolved. According to the figure then obtained the purity of the sample was 136 R.u./mg., indicating that not more than a quarter of the rennin had decomposed.

The stability of dried rennin crystals suggests that rennin might be useful for studies in which water usually interferes, as, for example, examination by infra-red rays for the detection of hydrogen bonds.

(*d*) *Proteolytic activity.* The purified and partly crystalline material from one of the preliminary preparations was used for these experiments. It has been shown that commercial rennin will digest haemoglobin (Hilton, 1941), and since this has already been adopted as a suitable substrate for pepsin it was chosen for rennin also. The results suggest that, had the earlier workers been able to control the pH of their enzyme-protein mixtures, the controversy over the identity or otherwise of pepsin and rennin would not have arisen. Text-fig. 3 indicates clearly that pepsin has been largely or entirely removed during the purification of the rennin, and that commercial rennet is a mixture of rennin, having an optimum pH of about 3·7 for its action on haemoglobin, and pepsin, with an optimum at pH 1·8. Text-fig. 3 also confirms the



Text-fig. 3. Proteolytic activity of rennin, commercial rennet and commercial pepsin on haemoglobin at various pH values.

generally held, though not previously proven, view that rennin is a protease. The optimum pH is rather different from that of pH 4·7 given by Davis & Davies (1934) for the protease of commercial rennet. This is probably due partly to their use of casein as a substrate, although the type of rennet they used and the conditions chosen for proteolysis (42 hr.–18 days as against 20 min.) may have exerted some effect.

Oppenheimer (1936) has pointed out the possibility that rennin consists largely of a cathepsin liberated by the breakdown of the cells of the mucosa during extraction. That it is actually secreted is shown by the results obtained by Berridge, Davis, Kon, Kon & Spratling (1943) on stomach juice removed through a fistula from a living calf (cf. Fomin, 1939). The activated juice had a proteolytic activity on haemoglobin, and an optimum pH and ratio to milk-clotting activity similar to that of purified rennin. It is possible that rennin, acting at pH 3·7, corresponds to the human cathepsin with an activity at pH 4·7 reported by Freudenberg & Buchs (1940). Unlike the cathepsins, it is not activated by H_2S (Hilton, 1941), and, according to Moriyama & Ōhasi (1941), is rather inhibited by H_2S and traces of heavy metals.

DISCUSSION

As already mentioned, the properties of crystalline rennin indicate that it may be an extremely useful member of the protein group for the study of certain properties. Its low solubility and its stability on drying should facilitate the solution of solubility and structural problems, while an examination of its hydrolytic activity towards synthetic substrates (cf. Bergmann & Fruton, 1941) would probably provide further interesting examples of enzyme specificity.

It is interesting to note how the history of work on rennin is similar to that of work on pepsin. Just as in the case of pepsin we have evidence for the existence of both an enzymically active protein and a non-protein enzyme, so with rennin; the results reported here, as well as those of other workers (e.g. Hankinson, 1943; Tauber & Kleiner, 1932), support the theory that rennin is a nitrogenous substance, while Lüers & Bader (1927), and Rao, Rao, Ramaswamy & Subrahmanyan (1941) present evidence to the contrary. The solution of the apparent contradiction necessitates further work, possibly along lines similar to those of Kraut & Tria (1937), who prepared the two types of pepsin from the same source, going further, if possible, with the preparation of the nitrogen-free enzyme from the pure nitrogenous material. If this is impossible, evidence could perhaps be obtained to show whether both substances were originally present in calf gastric fluid, or whether the non-nitrogenous material is present in the cells of the mucosa as a precursor of the zymogen which is actually secreted. This might lead to important discoveries concerning the synthesis of enzymes by glandular tissue.

SUMMARY

1. Experiments culminating in the crystallization of rennin are described.

2. Evidence that the crystals are pure rennin is presented.

3. Rennin crystals are of very low solubility in water and can be dried without decomposition.

4. Rennin is probably a protease, acting optimally on haemoglobin at pH 3·7.

The author wishes to express his gratitude to Benger's Ltd., and Messrs B. D. Thornley and S. Hilton, without whose aid this work could not have been carried out, to Dr J. G. Davis and Prof. H. D. Kay of this Institute for their kind interest and encouragement, and to Mr N. J. Gruber for photographing the rennin preparations.

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Physical Properties of Bovine Serum after Heating with Formaldehyde

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Several workers have tried bovine serum as a substitute for human plasma for transfusion, and in most cases serious reactions were observed (Edwards, 1944). Untreated bovine serum is antigenic and contains agglutinins for all groups of human red cells up to a dilution of 1/16. Cohn (1942) found that human and bovine serum albumins have similar crystal form and physical properties. The transfusion of bovine albumin, however, causes violent reactions though all agglutinins have been removed (Edwards, 1944). Any satisfactory substitute for human plasma must be non-toxic, non-antigenic, free from agglutinins and must have a colloid osmotic pressure similar to that of human plasma.

Since bovine serum is a convenient starting material, attempts were made to modify it so as to make it suitable for transfusion; this was done by heating it with formaldehyde and ammonia. The clinical results obtained with this material have been reported by Edwards (1944),* and an account is now given of its physical properties. It may be recalled that Van der Scheer, Wyckoff & Clarke (1941) found the formation in heated serum of a complex of large molecular weight migrating electrophoretically with a velocity intermediate between those of albumin and β-globulin. Davis, Hollander & Greenstein (1942) showed that the effects of ultra-violet light are similar to those of heating, but more slowly produced. Kleczkowski (1941*a*,*b*,*c*) discusses the antigenic properties of complexes formed by heating mixtures of proteins and the effect of heat on antibodies.

* The reporting, in this paper, of the ultracentrifuge results obtained by one of the present authors at Oxford was done without his knowledge or approval.

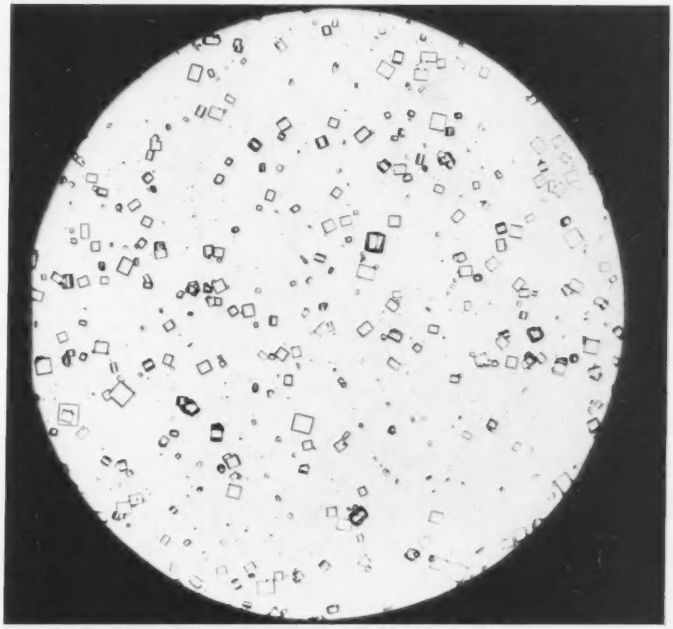

Fig. 1. Rennin crystals, × 300 (fraction 2).

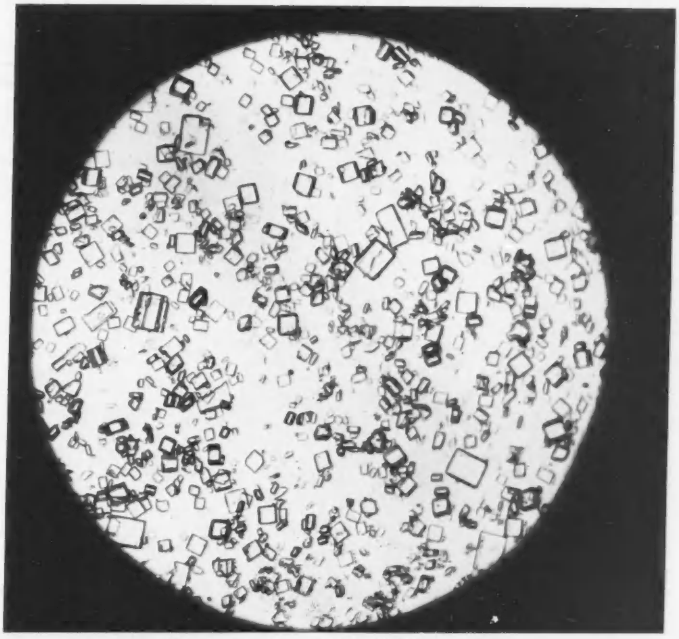

Fig. 2. Rennin crystals, × 300 (fraction 1).

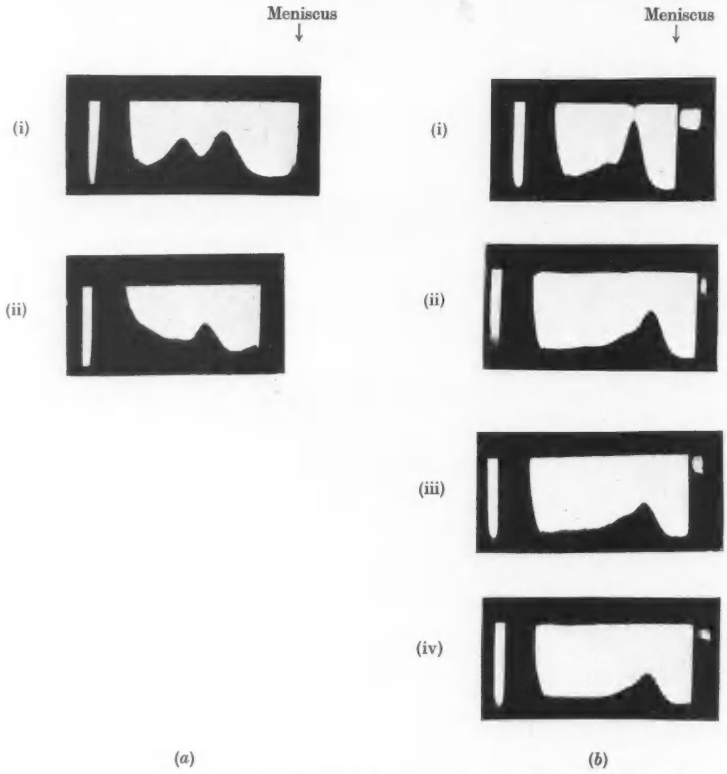

Fig. 1. Ultracentrifugal experiments. (*a*) (i) normal serum and (ii) serum heated 80° for 30 min., 1 hr. 30 min. after start of the run. The ordinate magnification is 2·2 times greater in (ii) than in (i). (*b*) Serum heated for 30 min. at (i) 50°, (ii) 60°, (iii) 65°, (iv) 70°, 1 hr. 20 min. after start of the run. Same ordinate magnification throughout.

Fig. 2. Electrophoretic experiments. (*a*) Normal serum (1 hr. 25 min. after start of the run). (*b*) Serum heated at 73° for 10 min. (1 hr. 25 min. after start of the run). (*c*) Serum heated at 73° for 30 min. (1 hr. 30 min. after start of the run).

EXPERIMENTAL

(1) *Preparation of material.* Bovine serum was obtained by drawing serum off the clot. It was then either passed through a bacterial filter and stored in sterile bottles, or stored frozen at $-12°$.

Serum coagulates on heating at 70° for 10 min. To prevent this, 0·2% by volume of 40% (w/v) formaldehyde was added before heating, and after mixing, a volume of ammonia (sp.gr. 0·88) equal to that of the formaldehyde was added to convert excess of the latter to hexamethylenetetramine. All the heated sera referred to below were treated in this manner. The heating was performed by placing small vessels containing the serum in a water-bath of the required temperature. The serum reached this temperature in 1 min. (included in the stated times of heating). After the allotted period, the samples were cooled rapidly. In some experiments the serum was diluted before heating.

(2) *Agglutination.* Heating at 73° for 10 min. was found to destroy all the agglutinins. At 65°, on the other hand, Edwards (1943) found that 3 hr. heating was required.

(3) *Osmotic pressure.* Osmotic pressures were measured by the method of Adair (1925) in collodion membranes containing about 2 ml., and dialyzed against physiological saline, the samples of serum being diluted with saline to a protein content of 4%. The results were as follows:

Sample	Osmotic pressure (mm. H_2O)
Untreated serum	190
Serum + formaldehyde and ammonia	190
Heated at 50° for 30 min.	180
Heated at 60° for 30 min.	140
Heated at 65° for 30 min.	120
Heated at 70° for 30 min.	110
Heated at 80° for 30 min.	90

(4) *Ultracentrifuge measurements.* Sedimentation was carried out in the Svedberg oil-turbine ultracentrifuge at a speed of 1000 rev./sec. and observed by the diagonal schlieren method of Philpot (1938). Two sets of measurements were made. In the first, normal bovine serum was compared with serum heated at 80° for 30 min., the samples being diluted with 0·2M-NaCl to give a protein concentration of 1%. The amounts of material appearing in the boundaries were then compared with the total refractive material of the untreated serum.

Sample	s_{20} (uncorrected) $\times 10^{-13}$		Fraction appearing in boundaries	
	Albumin	Globulin	Albumin	Globulin
Untreated serum	3·46	6·21	0·40	0·39
Heated at 80° for 30 min.	4·13	—	0·10	0

In the second set, the samples heated at 50, 60, 65 and 70° and used for osmotic measurements were compared; in each case the treated serum was diluted six-fold with 0·2M-NaCl. The amounts of material appearing in the boundaries are expressed as fractions of the total refractive material of the 50° sample, after it had been cleared by centrifuging.

Sample	s_{20} (uncorrected) $\times 10^{-13}$		Fraction appearing in boundaries		
	Albumin	Globulin	Albumin	Globulin	Total
50°	4·14	6·12	0·71	0·23	0·94
60°	4·39	—	—	—	0·93
65°	4·30	—	0·66	—	0·66
70°	4·30	—	0·48	—	0·48

Pl. 2, fig. 1 gives examples of the sedimentation diagrams obtained. The sedimentation constant of the albumin fraction is somewhat increased by the mildest treatment, but undergoes no further change; the sedimentation constant of the globulin component is unchanged. The amounts of both components undergo a progressive decrease as the severity of the treatment is increased; the globulin is more easily affected, not being visible as a separate component after heating above 60°; the albumin is still visible after heating at 80°, though greatly decreased in amount.

(5) *Electrophoresis.* Samples of untreated serum and serum heated at 73° for 10 and 30 min. were compared in the electrophoresis apparatus described by Tiselius (1937) and the moving boundaries were observed by the method of Svensson (1939, 1940). The samples were dialyzed against phosphate buffer of pH 8·0 and ionic strength 0·1 and diluted with the same buffer to about 2% with respect to protein. Pl. 2, fig. 2 shows the electrophoresis diagrams obtained.

The untreated serum showed the same components as described by Cohn (1941). The heated samples differ from the untreated in showing no resolution into components, though the migrating material is rather heterogeneous.

(6) *Viscosity.* Viscosity measurements were carried out on samples prepared from a stock serum of protein content 8%. The rotating cylinder method described by Robinson (1939) was used. This method, by alteration of shear rate, differentiates between absolute viscosity due to the volume of the particles in solution and structural viscosity due to asymmetry of the particles. Untreated serum showed a slight structural viscosity. Addition of formaldehyde and ammonia, without heating, markedly increased the absolute viscosity. This effect has been noted by Foster & Biguria (1943), who find that it is due to the effect of formaldehyde on the globulins. Subsequent heating caused a loss of the small structural viscosity effect, but a progressive increase of the absolute viscosity.

Sample	Viscosity/viscosity of water (13°)
Untreated serum	2·52–2·88
Serum with formaldehyde and ammonia	3·21–3·32
Heated at 70° for 10 min.	7·35
Heated at 70° for 20 min.	17·3
Heated at 70° for 30 min.	32·1

DISCUSSION

All these results are consistent with a progressive alteration of the serum proteins by heating with formaldehyde and ammonia. There is an increasing heterogeneity with respect to particle size and to electrophoretic mobility; the heterogeneity accounts for the 'disappearance' of the fractions as seen in the sedimentation diagrams. The fall of osmotic pressure shows that the mean particle size increases. The increase of viscosity is, however, out of proportion to the increase in particle size; the absence of structural viscosity in the heated samples shows that the abnormal rise in viscosity cannot be due to a greatly increased asymmetry of the particles and it must, therefore, be ascribed to an increased solvation of the altered particles. This applies only to that fraction of the protein which does not appear in the sedimentation diagrams, since the sedimentation constants of the recognizable components are little changed.

SUMMARY

1. Bovine serum heated with formaldehyde and ammonia over a range of conditions shows loss of specific properties and a progressive aggregation of its proteins to form a polydisperse system of larger particles.

2. The osmotic pressure, viscosities and electrophoretic and ultracentrifugal characteristics of a range of samples have been measured.

We are indebted to Dr G. Weber for his help and advice with the viscosity measurements.

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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

2. THE EFFECT OF TISSUE EXTRACTS

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We have already described experiments on the nature of some of the factors which influence the growth of fibroblasts *in vitro* (Davidson & Waymouth, 1943). In these experiments fibroblast cultures were grown in roller tubes according to the technique of Willmer (1942), and the amount of growth was assessed by making chemical estimations of the nucleoprotein phosphorus (NPP) content of the cultures. In this paper the original observations have been continued and extended.

The technique of estimating the growth of tissue cultures by measuring the increase in nucleoprotein phosphorus (NPP) in the growing tissue was introduced by Willmer (1942) and was recommended by Cunningham & Kirk (1942). Willmer presumed that such an estimation might give a measure of the amount of nuclear material present, but he pointed out that the presence of nucleic acid as a cytoplasmic constituent in certain tissues was a possible drawback to the method. We have recently shown (Davidson & Waymouth, 1944 *a*, *b*) that mammalian and avian tissues contain two types of nucleic acid, deoxyribonucleic acid in the nucleus and ribonucleic acid in the cytoplasm and that in many tissues the ribonucleic acid is the greater in amount. In liver, for example, some 60–80% of the total nucleic acid is present as the cytoplasmic ribonucleic acid (Davidson & Waymouth, 1944*c*). In the rapidly

growing tissues of the embryo the total nucleic acid content is greater than in the corresponding adult tissue, but for any given tissue the ratio of ribonucleic acid to deoxyribonucleic acid is of the same order in the embryo as in the adult. This ratio is not known with any degree of certainty for connective tissue or fibroblasts. It can, however, be presumed that NPP which includes both ribonucleic acid and deoxyribonucleic acid will give a reasonably accurate measure, in any one type of tissue, not of nuclear material but of total cellular material (cf. Carruthers & Suntzeff, 1942), and that it can legitimately be used for the assessment of the growth of tissue cultures.

It must be emphasized that any tissue-culture method for the investigation of the tissue growth-promoting activity of a substance is relatively crude in comparison with the recognized methods for examining growth-promoting factors for yeast or many types of bacteria. In the case of many micro-organisms the investigator can start with a basal medium of known composition containing the necessary known growth-promoting factors. The growth response with such a medium under standard conditions is dependent on the concentration of the factor under test. In a tissue-culture test such simple conditions are not possible. In the first place, growth occurs in a two-phase system consisting of plasma clot and fluid medium. The plasma varies in composition from bird to bird and contributes unknown factors to the medium, so that comparison of the growth-promoting activity of two media can be made only when a single batch of plasma is used throughout. Secondly, the growing tissue requires a large number of factors, nutrient and biocatalytic, whose nature is unknown. Thirdly, the tissue itself cannot be implanted in quantitatively accurate amounts as can a cellular suspension (of yeast or bacteria) be used to seed a medium. And unless a pure strain of cells is available (and it was not in the present investigation), the tissue used for the original explants will be variable in composition and in potential growth capacity. For these reasons the present investigation is aimed at detecting only gross differences in the growth-promoting power of the materials tested.

METHODS

Fresh explants of the 9-day chick embryo heart were grown in plasma clot in roller tubes by the method previously described (Davidson & Waymouth, 1943, 1944*d*). For the first 2 days the fluid phase in the roller tubes consisted of Tyrode solution alone. At the end of this period the Tyrode was replaced by the test solution, and the tubes allowed to rotate for a further 2 days. The NPP in the tissue was then determined as previously described (Davidson & Waymouth, 1943). The change in NPP during the 2 days of the test is taken as a measure of the amount of growth which has occurred.

In agreement with results previously recorded, it was found that when the fluid phase during the 2 days of the test proper consisted of Tyrode solution alone, a slight fall in NPP occurred. When the fluid phase contained embryo extract, the NPP rose. These observations have a bearing on the method by which the results are best expressed (*vide infra*).

Preparation of embryo extract. The preparation of sterile chick embryo extract has already been described (Davidson & Waymouth, 1943).

In winter it has been possible to obtain large quantities of sheep embryos 9–10 weeks old. Extract was prepared by mincing the embryos and allowing the pulp to stand overnight in the refrigerator with an equal volume of water, Tyrode solution or 0·9 % NaCl. The extract was then filtered through muslin and centrifuged. It was then sterilized, usually by the use of large Berkefeld candles. The extracts are viscous and tend to clog the candles easily, but with candles of a large surface area, sufficient sterile material could be obtained before the candles became clogged. A loss of about 40 % total N in the extract resulted from filtering thus in some cases. The sterile extract was suitably diluted with sterile Tyrode solution before use.

In a few cases sterilization was avoided by preparing the extract aseptically from very small embryos. The complete uterus was brought from the slaughter-house to the laboratory and opened aseptically. The embryo was transferred to a sterile glass vessel and minced with sterile scissors. An equal volume of sterile Tyrode solution was added and the mixture centrifuged. The sterile '50 % embryo extract' was then pipetted off and stored in sterile tubes.

Method of expressing results. The results of a series of ten individual experiments with chick embryo extract (nitrogen concentration = 28–31 mg./100 ml.) (N = 30)* are shown in Table 1, arranged in decreasing order of initial

Table 1. *Increase in NPP in tissue grown in embryo extract* (N = 30) *during 2-day test period*

(Plasma blanks subtracted.)

Test no.	NPP content: Initial (μg.)	Final (μg.)	Increase (μg.)	Relative increase
25	1·53	1·96	0·43	0·28
45	1·50	1·91	0·41	0·27
44	1·34	1·71	0·37	0·28
49	1·23	1·68	0·45	0·36
56	1·14	1·56	0·42	0·37
82	1·12	1·44	0·32	0·29
61	0·90	1·29	0·39	0·43
69	0·89	1·22	0·33	0·37
75	0·89	1·33	0·44	0·49
67	0·86	1·23	0·37	0·43
Mean and standard error			0·39 ± 0·014	0·36 ± 0·024

NPP values. These initial values cover a wide range (0·86–1·53 μg.). The increase in NPP in the course of 24 hr. is expressed in two ways: (i) as an absolute increase per roller tube, and (ii) as a relative increase $\frac{\text{Final NPP} - \text{Initial NPP}}{\text{Initial NPP}}$.

* This convention will be used to express the concentration of total N in mg./100 ml. in the 0·5 ml. fluid added to the roller tubes at the beginning of the test period.

These ten tests involved the use of 35 roller tubes, i.e. 215 pieces of tissue with the corresponding plasma blanks. It can be seen (Table 1) that there is a smaller range of variation in the increases in NPP expressed as an absolute increase than in those expressed as a relative increase. The effect of embryo extract therefore seems to be to cause, during the 2-day growth period, an increase in NPP which is not proportional to the amount of tissue initially present. The limiting factor for growth lies, under these conditions, in the medium and not in the tissue.

Individual figures for a series of experiments, in which tissue is grown with Tyrode solution alone as fluid phase, are shown in Table 2, again arranged in descending order of initial NPP values. In this case the change in NPP (usually a fall, Davidson & Waymouth, 1943) is greater for higher initial values of NPP. Where the initial figures are high ($>1{\cdot}0\,\mu$g.) the absolute differences are greater, and where the initial figures are low ($<1{\cdot}0\,\mu$g.) the absolute differences are less than the relative differences. Here the tissue itself, in the absence of a growth-promoting medium, controls the magnitude of the change in NPP.

Table 2. *Change in NPP in tissue grown in Tyrode solution alone during 2-day test period*

(Plasma blanks subtracted.)

Test no.	NPP content: Initial (μg.)	Final (μg.)	Change (μg.)	Relative change
33	1·75	1·29	−0·46	−0·26
39	1·70	1·19	−0·51	−0·30
45	1·46	1·38	−0·08	−0·05
44	1·42	1·01	−0·41	−0·29
50	1·36	1·25	−0·11	−0·08
47	1·35	1·34	−0·01	−0·01
48	1·25	1·08	−0·17	−0·14
46	1·22	1·22	±0	±0
38	1·20.	1·25	+0·05	+0·04
55	1·20	0·92	−0·28	−0·23
91	1·19	1·14	−0·05	−0·04
68	1·12	0·99	−0·13	−0·12
42	1·06	1·20	+0·14	+0·13
41	1·04	1·10	+0·06	+0·06
34	0·99	0·99	±0	±0
61	0·86	0·79	−0·07	−0·08
67	0·78	0·70	−0·08	−0·10
69	0·56	0·59	+0·03	+0·05
60	0·55	0·44	−0·11	−0·20
Mean and standard error			−0·11±0·04	−0·08±0·03

It has therefore been concluded that the absolute increases in NPP per roller tube during the growth period express satisfactorily the effects of growth-promoting media derived from embryonic tissue, and it has been assumed that the method is valid for other types of growth-promoting medium. It should be emphasized that the differences in initial NPP values are, of course, never so gross in a single test as in the range shown in Table 2.

In the experiments recorded here, large differences are sought and a positive change of $>0{\cdot}2\,\mu$g./roller tube in the 2-day period is taken as indicating definite growth-promoting properties in the medium under test. A rise of $>0{\cdot}5\,\mu$g. indicates very good growth. With each medium tested at least two, and in most cases three or more, roller tubes are used at each time, i.e. the number of pieces of tissue is at least 12 and in most cases is 18 or more. The number of tubes which can be carried on the drum is limited to 20 for any one test. Plasma blanks were therefore dispensed with in some experiments, after it had been established that the correction was insignificant for the preparations under test.

RESULTS

(1) *Comparison of chick embryo extract with sheep embryo extract* (Table 3, tests 128/1 and 130/1)

A comparison was made of the growth-promoting power of chick and sheep embryo extracts. Both were prepared aseptically from sterile embryos (i.e. sterilization was avoided) by mixing the embryo pulp with an equal volume of Tyrode solution and centrifuging. The '50%' chick embryo extract prepared in this way had a total N content of 60–90 mg./100 ml., while '50%' sheep embryo extract contained 200–300 mg. N/100 ml. They were both diluted to the same nitrogen content and tested at N = 20, 30 and 50. Chick embryo extract was more effective under these conditions than sheep embryo extract.

(2) *The effect of drying*

Sheep embryo extract was dried by a number of methods: (i) Portions of 30–50 ml. were dried in the frozen state in an apparatus similar to that described by Christensen (1942) and by Hays & Koch (1942). The dried product dissolved completely in water ('Frozen-dried extract'). (ii) A large batch of about 5 l. was sent in a refrigerated container to the Low Temperature Research Station at Cambridge where it was dried from the frozen state in a large-scale apparatus. It was dissolved from the walls of the apparatus in a small amount of water and the solution was dried in vacuum desiccators. A large part of the product did not dissolve in water ('Cambridge-dried extract'). (iii) A large amount of material was dried at room temperature in thin layers in Petri dishes in vacuum desiccators over concentrated sulphuric acid. The dry product was scraped off the dishes and finely powdered. A large proportion was insoluble in water ('Vacuum-dried extract'). (iv) Thin layers of extract dried at room temperature in flat dishes in the blast of an electric fan according to the method of Fischer (1941*b*) gave a similar material containing a large amount of insoluble matter.

In most cases the dried material was suspended in water, the insoluble residue centrifuged off, the chloride content determined, and the salt concentration corrected by addition of suitable amounts of double-strength Tyrode solution. The reconstituted extract was diluted to a suitable total N con-

centration with ordinary Tyrode solution and sterilized by passage through a small filter candle. In some cases the dried extract was reduced to a fine powder by grinding and sieving and was extracted with benzene to remove lipid materials before being reconstituted.

Small portions (50–100 mg.) of the dry powder were also sterilized by allowing them to stand for some days under benzene in sterile, plugged 15 ml. conical centrifuge tubes (cf. Fischer, 1941*b*). The bulk of the benzene was then poured off and the plugged tube placed in a vacuum desiccator to remove the last traces of benzene. The sterile powder was then suspended in sterile distilled water, allowed to stand 1–2 hr. and the insoluble residue centrifuged down. This treatment not only sterilized the powder but also removed lipid material from it.

An attempt was made to dry embryo extract in the spray-drier described by Edwards, Kay & Davie (1940), but even a '50% extract' contained insufficient solids to yield satisfactory results. Attempts at spray-drying at the Low Temperature Research Station in Cambridge were also unsuccessful.

Examples of some results obtained with dried embryo extracts are shown in Table 3 (tests 148/1, 95/1, 98/1).

Chick embryo extract, when dried in the frozen state, sterilized under benzene, and reconstituted, gave an extract which was more potent than the original fresh material at the same N concentration (test 148/1). The increase in activity may be due in part to the removal of inhibitory lipid materials by the benzene (*vide infra*).

Sheep embryo extract, 'vacuum-dried' or 'Cambridge-dried', gave preparations which contained a large amount of insoluble material. The activity of the solutions from these powders was low but was enhanced by previous extraction of the dried powder with benzene (*vide infra*) (tests 95/1, 98/1).

(3) *The effect of defatting*

(i) *By ether.* The removal of lipid material from liquid embryo extract was carried out by freezing with ether according to the method used by McFarlane (1942) for serum. Popják & McCarthy (1943) have shown that this treatment removes from serum all the cholesterol, approximately two-thirds of the phospholipids and two-thirds of the neutral fats.

100 ml. sheep embryo extract were shaken with 30 ml. ether and frozen to a temperature below −25° by immersion of the flask in a solid CO_2-ethanol mixture. Freezing and thawing were carried out rapidly, and a top layer containing fat and some denatured protein was obtained. The clear lower aqueous layer was sucked off and the extraction with ether repeated. The clear extract was freed

Table 3. *Comparison of the growth-promoting effects of fresh, dried, defatted and stored embryo extracts*

Test no.	Species	State	Defatted	Sterilized	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
128/1	Chick	Fresh	—	Prepared aseptically	30	+0·44
	Sheep	,,	—	,,	30	+0·24
130/1	Chick	,,	—	,,	50	+1·00
	,,	,,	—	,,	20	+0·37
	Sheep	,,	—	,,	50	+0·46
	,,	,,	—	,,	20	+0·16
95/1	Sheep	Vacuum-dried	—	Berkefeld candle	—	+0·18
	,,	,,	—	,,	—	−0·05
	,,	,,	Benzene	Benzene	—	+0·36
98/1	Sheep	Cambridge-dried	—	Berkefeld candle	9	+0·11
	,,	,,	Benzene	,,	9	+0·18
127	Sheep	Fresh	—	,,	30	+0·23
	,,	Frozen-dried	Ether	L3 candle	30	+0·58
	,,	,,	,,	Berkefeld candle	30	+0·48
129/1	Sheep	Fresh	—	,,	40	+0·29
	,,	Frozen-dried	Ether	,,	40	+0·61
148/1	Chick	Fresh	—	Prepared aseptically	20	+0·31
	,,	Frozen-dried	Benzene	Benzene	20	+0·60
151/2	Chick	Fresh	Ether	Prepared aseptically	50	+1·16
125/1	Sheep	Stored sterile for 9 months at −30°	—	Berkefeld candle	30	+0·73
	,,	Stored 9 months at −30° and then sterilized	—	,,	30	+0·38
	,,	Prepared from embryos stored for 9 months at −30°	—	,,	30	+0·16

from ether in a vacuum desiccator, refrozen, thawed and filtered through paper. In one instance the total N of sheep embryo extract before defatting was 247·5 mg./100 ml. and phospholipid P was 1·41 mg./100 ml. After defatting, the total N was 210·0 mg./100 ml. and phospholipid P was negligible. Defatted extracts filtered easily through a Berkefeld candle, but it was difficult to remove the last traces of ether from them. Such extracts which had been dried in the frozen state (and therefore completely freed from ether) and reconstituted were more active than fresh extract at the same N concentration (Table 3, test 127).

The results of a test with chick embryo extract defatted by freezing with ether under aseptic conditions (i.e. sterilization of the defatted solution was unnecessary) are shown in Table 3, test 151/2. The preparation had good growth-promoting activity, though a direct comparison with ordinary chick embryo extract at the same N concentration was not made (but cf. test 130/1) (see also § 6, and Table 4, test 90).

(ii) *By benzene.* As we have already shown, extraction of dried embryo extract with benzene before reconstitution enhanced the activity of the preparations (Table 3, tests 95/1, 98/1).

(4) *Extracts dried after defatting* (Table 3, test 125/1)

Extracts from which lipid material had been removed by freezing with ether as described in § 3 (i) were also dried from the frozen state. When an extract was defatted in this way, frozen-dried and reconstituted, the powder dissolved very readily to give a solution which filtered easily through a candle and had high activity (Table 3, tests 127, 129/1). The most active dry preparations of sheep embryo extract were made in this way.

(5) *Effect of storage on embryo extract*

The effect of storage on the growth-promoting properties of sheep embryo extract was examined. Storage was carried out for 9 months in a cold chamber at −30° in the Torry Research Station. Sterile sheep embryo extract, sealed up in ampoules and stored, retained powerful activity after 9 months. When embryo extract was stored in the non-sterile state and Berkefeld-filtered after storage for 9 months, the activity was relatively good. When whole embryos were stored for 9 months and then converted into extract and filtered, the extract was still less active (Table 3, test 125/1). It is preferable, therefore, to store embryo extract in the sterile state in sealed ampoules.

(6) *Comparison of the growth-promoting properties of extracts from various organs and tissues of the embryo*

(i) *Sheep embryos.* Extracts were prepared by mincing the different embryonic organs finely in a large Latapie mincer, adding 1 vol. Tyrode solution, allowing the mince to stand overnight at 0° and centrifuging. The extracts were sterilized by filtration and tested at the same N concentration (Table 4, tests 39, 36, 83, 84/1).

A test was also made with extracts which had been defatted by freezing with ether (Table 4, test 90). None of the extracts of individual tissues gave a better response than whole embryo extract. Extracts of muscle, heart and cartilage were possibly slightly superior to those of viscera, e.g. liver.

(ii) *Chick embryos.* Nine-day chick embryos were too small for the preparation of extracts of individual organs. Aseptic dissection of embryos 12 or 13 days old was possible, and extracts of muscle, cartilage, brain, viscera and of the carcass from which brain and viscera had been removed, were prepared and tested at the same N concentration (N = 20). The results were not completely conclusive, but they did not indicate that the activity of chick embryo extract as a growth-promoting medium could be predominantly attributed to any particular tissue.

(7) *Ethanol precipitates from tissue extracts*

(i) *From embryo tissue extracts.* Sheep embryo extracts were treated in the cold with 4 vol. of ice-cold ethanol. The precipitate was centrifuged

Table 4. *Comparison of the growth-promoting properties of extracts of various organs and tissues of the sheep embryo*

Test no.	N concentration of extract (mg./100 ml.)	Change in NPP content of cultures (μg.) induced by extract of: Whole embryo	Viscera	Brain	Cartilage	Heart	Liver	Muscle	Lung
39	42	—	+0·04	—	+0·29	—	—	+0·22	—
36	30	—	—	—	+0·26	—	—	—	—
83	10	—	—	—	—	—	+0·12	+0·31	—
84/1	10	—	—	±0·0	+0·04	+0·27	—	—	+0·14
90	Varied with the tissue used; '50% extracts' diluted 1 in 4	+0·51* +0·74†	—	+0·45†	—	+0·40†	+0·12†	+0·44†	—

* Extracts defatted by freezing once with ether.

† Extracts defatted by freezing twice with ether.

down quickly and dried *in vacuo* over $CaCl_2$. In some cases the precipitate was washed with cold absolute ethanol and ether before being dried. The dried precipitates were ground finely and extracted once with ether. Powders were made in this way from individual minced sheep and calf embryo organs and also (*vide infra*) from adult sheep heart by extracting with 1 vol. of 0·9% NaCl and precipitating the extracts with ethanol. Such ethanol powders were found to be only slightly soluble in water.

(*a*) The powders were allowed to stand in 100 mg. portions with 10 ml. Tyrode solution for 4 hr. at 0°. The suspensions were then centrifuged and the supernatant fluids passed through L3 Pasteur-Chamberland candles. Total N was determined in the sterile extracts. Such extracts from embryo brain, heart, liver and muscle powders were found to contain 13, 21, 28 and 39 mg. N/100 ml. respectively.

(*b*) To obtain a more concentrated solution and to sterilize the solution without the use of filter candles, portions of these ethanol powders were dissolved in water with the aid of heat, and the supernatant fluid obtained on centrifuging was sterilized by heating to 100° for 10 min. After cooling, 1 vol. of double-strength Tyrode solution was added.

(*c*) To avoid the use of candles or of heat in obtaining a sterile preparation, portions of 50 mg. of the ethanol powders were transferred with the aid of a sterile platinum scoop (Packchanian, 1942) to a sterile 15 ml. pyrex centrifuge tube which was then filled with Analar benzene, stoppered with a sterile plug of cotton-wool and allowed to stand for several days. The benzene was decanted off and the powder dried *in vacuo*. To the dry powder, now sterile, 5 ml. sterile Tyrode solution were added and the tube set aside for 4 hr. at 0° before being centrifuged. The supernatant fluid was analyzed for total N.

The results (Table 5, tests 130/2, 131, 133, 134) show that active preparations can be prepared from these ethanol precipitates both from whole sheep embryo extract and from extracts of individual organs. Extracts of brain and muscle were superior to extracts of liver (test 133). The results obtained with preparations heated or sterilized by benzene were similar to those from Berkefeld-filtered preparations.

Precipitation of sheep embryo extract with acetone in place of ethanol was also tried. Ice-cold sheep embryo extract was treated with 4 vol. ice-cold acetone and the precipitate immediately centrifuged down and dissolved in ice-cold water. Almost the whole precipitate dissolved, yielding a reddish solution with no trace of the brown colour which develops when the operation is carried out at room temperature. The solution was frozen-dried and a fluffy powder obtained. It was freely soluble in water and was sterilized by a Berkefeld candle. The solution had no activity.

(ii) *From adult and embryo sheep heart extracts.* Extracts of adult heart tissue from the fowl (Doljanski & Hoffman, 1939; Hoffman, Tenenbaum & Doljanski, 1939, 1940) and from the sheep, cow, rabbit and dog (Doljanski, Hoffman & Tenenbaum, 1942) (but not those from the rat (Hoffman, 1940) unless lipid material is removed from the tissue before preparation of the extract (Werner, 1944)), including ethanol precipitates from such extracts (Werner & Doljanski, 1942; Werner, 1944) are believed to exert a growth-promoting action on tissue cultures, and these extracts and precipitates have recently been applied to the healing of wounds (Doljanski & Auerbach, 1944; Auerbach & Doljanski, 1944; Mandl & Maybaum, 1943; Mandl, 1944; Hoffman & Dingwall, 1944; Kerr & Werner, 1944). We have accordingly prepared extracts and precipitates from adult sheep heart by the method described by Werner & Doljanski (1942), and by Kerr & Werner (1944).

Fresh adult sheep heart was finely minced, extracted with 5 vol. of physiological saline solution and the extract precipitated with 4 (in some batches 2) vol. of ethanol. The precipitate was centrifuged down and dried *in vacuo* over $CaCl_2$. The dry powder was extracted with ether and dried again. The amount of material which could be brought into solution from the adult heart powders was even less than from embryo tissue powders. Extracts from powders prepared in this way were compared with extracts from powders prepared in the same way from sheep embryo heart and whole sheep embryo extract, using the methods described in (i) above. The results are shown in Table 5, tests 130/2, 131, 132/1, 135, 137, 139/1.

In general, preparations from adult sheep heart were inferior to preparations of the same N concentration from embryo sheep heart and to the corresponding preparations from whole embryo extract. In one instance (test 137), where such preparations were compared at low N concentrations (N = 6), the adult sheep heart appeared to be more active.

In order to obtain a more soluble preparation from the ethanol precipitate of adult sheep heart extract, we extracted 10 g. of the dry powder with 100 ml. 0·01 N-ammonia as recommended by Werner & Doljanski (1942). After standing at room temperature for 15 hr., the insoluble residue was centrifuged down and the extract dried in a vacuum desiccator. The resulting material (270 mg.) was powdered and sterilized under benzene. It dissolved fairly readily in sterile Tyrode solution. After a small amount of insoluble material had been re-

Table 5. *Comparison of the growth-promoting properties of ethanol precipitates from tissue extracts*

Test no.	Tissue of origin	Method of sterilization	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
130/2	Adult sheep heart	L3 candle	20	+0·11
	Embryo sheep heart	,,	24	+0·37
131	Whole sheep embryo	L3 candle	36	+0·33
	,,	,,	14	+0·11
	,,	Heated	36	+0·33
	Whole chick embryo	L3 candle	1	−0·03
	,,	Heated	8	−0·14
	Adult sheep heart	L3 candle	14	+0·07
	,,	Heated	7	+0·05
132/1	Whole sheep embryo	L3 candle	25	+0·17
	,,	,,	25	+0·20
	Adult sheep heart	,,	25	+0·09
	,,	,,	25	+0·01
	,,	,,	45	+0·15
133	Sheep embryo brain	L3 candle	13	+0·54
	Sheep embryo heart	,,	13	+0·25
	Sheep embryo liver	,,	13	+0·18
	Sheep embryo muscle	,,	13	+0·24
	Sheep embryo heart	,,	21	+0·44
	Sheep embryo liver	,,	21	+0·22
	Sheep embryo muscle	,,	21	+0·55
134	Whole sheep embryo	Benzene	12	+0·57
	,,	L3 candle	12	+0·14
	Sheep embryo brain	,,	12	+0·17
	Sheep embryo heart	,,	12	+0·11
	Sheep embryo muscle	,,	12	−0·02
	,,	,,	35	+0·36
135	Whole sheep embryo	Benzene	25	+0·23
	Adult sheep heart	,,	25	+0·06
	,,	,,	46	−0·02
137	Adult sheep heart	Benzene	6	+0·45
	Whole sheep embryo	,,	6	+0·35
	Embryo calf muscle	,,	8	+0·41
	Embryo calf heart	,,	8	+0·24
	Embryo calf brain	,,	8	+0·21
139/1	Adult sheep heart	Benzene	8	+0·04
	Embryo sheep heart	,,	44	+0·31
	Whole sheep embryo	,,	30	+0·17
	Embryo calf muscle	,,	16	+0·25
159/1	Adult sheep heart—fraction soluble in dilute ammonia	Benzene	30	+0·44

moved at the centrifuge, the supernatant fluid was tested at N = 30 (Table 5, test 159/1). It had moderately good growth-promoting power.

(8) *The nucleoprotein fraction of embryo extract*

In view of the statements by Fischer (1939, 1940, 1941*b*) that growth-promoting activity is associated with the nucleoproteins of embryo extract, we have prepared the nucleoprotein fraction by the method of Hammarsten (1920) as employed by Fischer (1941*b*). This fraction has been termed 'embryonin' by Fischer & Astrup (1943).

'Cambridge-dried' sheep embryo extract (§ 2, ii; 3 g.) was suspended in 50 ml. ice-cold 0·06N-HCl and allowed to stand with occasional stirring in the refrigerator for 2 hr. The suspension was centrifuged and the supernatant fluid discarded. The residue was washed twice with ice-cold glass-distilled water. The residue was treated with a little ice-cold distilled water and, while cooled in ice, was treated with cold 0·06N-NaOH till the reaction was just alkaline (faint blue to litmus; pH < 9). Almost the whole residue dissolved. The solution while cooled in ice was then adjusted, with cold 0·1N-HCl, to pH 5·6–5·8, and the precipitate of nucleoprotein centrifuged down. Addition of another drop of HCl to the supernatant fluid gave no further precipitate. The nucleoprotein while cooled in ice was redissolved in water by cautious addition of 0·06N-NaOH, centrifuged, and the clear supernatant fluid (4·5 ml.) was dried from the frozen state. The dried material was a fluffy yellowish powder. It contained 10·96% N and 0·52% P.

The powder was treated with Tyrode solution and a small amount of insoluble material centrifuged down and discarded. The clear yellowish supernatant fluid could be sterilized by means of an L3 or small Berkefeld candle although some of the nucleoprotein was adsorbed by the candle. The sterile solution had good growth-promoting power (Table 6, test 125/2).

With most preparations, Berkefeld-filtration was avoided by sterilizing the powder under benzene. The sterile powder was then treated with sterile Tyrode solution, insoluble material was centrifuged down and discarded, and the sterile supernatant fluid was tested at various N concentrations (Table 6, tests 143, 148/2, 151/1). A typical solution contained 9·8 mg. N/100 ml. and 0·70 mg. organic P/100 ml. The proportion of P to N was thus higher in the solution than in the original powder. It had good growth-promoting power at low concentrations (N = 5). Higher concentrations (N = 12) appeared to be less effective (test 143). Hopkins & Simon-Reuss (1944) found that of two concentrations of hypoxanthine used, the lower was favourable to the growth of tissue cultures.

When estimations of tissue growth by means of NPP are made on cultures to which an amount of nucleoprotein corresponding to several μg. of NPP has been added, the question naturally arises, whether the increases in NPP observed might be due to adsorption of added nucleoprotein on to the plasma coagulum, and not to actual incorporation of the nucleoprotein in the living tissue. It was found that much higher concentrations of nucleoprotein than were used here were required to cause a significant rise in NPP in plasma blanks.

The nucleoprotein was tested also in conjunction with solutions of boiled kidney extract prepared by the method of Fischer & Astrup (1942), and with solutions of peptone. Both these solutions were sterilized by heat and have already been shown to have the power of causing a marked rise in the NPP of tissue cultures (Davidson & Waymouth, 1944*d*). The mixture of nucleoprotein and boiled kidney extract had very powerful growth-promoting power (Table 6, test 125/2). The addition of peptone was not so effective as the addition of kidney extract.

The nucleoprotein fraction prepared from liquid embryo extract by two methods was also tested. (i) *Greenstein's method.* The nucleoprotein fraction of fresh sheep embryo extract was prepared by the method of Greenstein & Jenrette (1940). This method is designed to avoid, as far as possible, protein denaturation during the isolation of the nucleoprotein fraction. The product which was nevertheless poorly soluble was dried from the frozen state, dissolved in Tyrode solution and Berkefeld-filtered. It had no activity. (ii) *Acid precipitation.* The precipitate obtained by acidifying embryo extract at 0° to pH 4·3–4·6 with acetic acid, when dissolved in the minimum amount of cold dilute alkali and sterilized by filtration after addition of an equal volume of double-strength Tyrode, had little or no activity.

Table 6. *The effect on the nucleoprotein phosphorus (NPP) content of tissue cultures of nucleoprotein prepared from dried sheep embryo extract by the method of Hammersten* (1920)

Test no.	Test substances	Method of sterilization of nucleoprotein	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
125/2	Nucleoprotein	L3 candle	10	+0·67
	"	Berkefeld candle	7	+0·41
	Nucleoprotein + boiled kidney extract	"	4+25	+1·58
	Boiled kidney extract	—	25	+0·97
143	Nucleoprotein	Benzene	12	+0·17
	"	"	4·5	+0·32
	Nucleoprotein + peptone	"	4·5+15	+0·52
	Peptone	—	15	+0·29
148/2	Nucleoprotein	Benzene	5	+0·42
	Nucleoprotein + peptone	"	5+7·5	+0·22
	Peptone	—	7·5	+0·24
151/1	Nucleoprotein	Benzene	5	+0·44
	Nucleoprotein + peptone	"	5+7·5	+0·57
	Peptone	—	7·5	+0·46

A considerable proportion of the total nitrogen of these nucleoprotein preparations was retained by the filter candles. Seitz filtration resulted in almost complete adsorption of the nucleoprotein on the filter pad.

To avoid the use of candles and filters which adsorb the active materials, preparations of nucleoprotein were also made aseptically by acid precipitation. To ice-cold sterile chick embryo extract, ice-cold dilute HCl (0·001–0·01 N) or acetic acid was added to give a pH of 4·3 and the precipitate centrifuged down. It was washed with sterile ice-cold water (slightly acidified) and dissolved in the minimum amount of ice-cold sterile 0·01 N-NaOH. All operations were, of course, conducted with sterile

glassware. To the neutral solution, 1 vol. of double strength Tyrode solution was added. It had no activity.

(9) *Histone*

In view of recent suggestions (Stedman & Stedman, 1943 *a*, *b*, *c*, 1944 *a*, *b*; Stedman, 1944) that the histone of the cell nucleus plays a part in controlling cellular proliferation, and particularly that a high content of histone 'produces an inhibition of both the processes which lead to mitosis and those which lead to the synthesis of chromosomin necessary for the duplication of the chromosomes' (Stedman & Stedman, 1943*c*), it was thought desirable to test the effect of histones on the growth of tissue cultures.

Histones from two sources were prepared by two different methods. (*a*) Thymus histone was prepared from calf thymus by the method of Felix & Harteneck (1926). The histone was free from phosphorus. (*b*) Nucleohistone was prepared from sheep spleen by the method of Mirsky & Pollister (1942). The histone was separated from the nucleic acid by the chloroform-octanol method of Sevag, Lackman & Smolens (1938).

solution was very small in all cases. In test 146, the N concentration was slightly higher than in other tests, owing to the presence of a small amount of histone in suspension.

In all cases histone alone had no effect on the NPP of the cultures. It was found that the addition of histone to embryo extract regularly produced a greater increase in NPP than did embryo extract alone. This effect was not an additive one, as the increases were generally greater than the sum of the NPP increases caused by embryo extract and by histone separately (Table 7).

DISCUSSION

It has been recognized since the early tissue culture work of Carrel (1913) that embryonic extracts can promote rapid growth of explanted tissue and can provide an adequate medium for prolonged growth *in vitro*. The form of test adopted here is designed to detect large and rapid increases, occurring within 2 days, in the nucleoprotein content of the growing fibroblasts. Fresh explants from the 9-day chick embryo heart have been used, and no attempt has

Table 7. *Effect of histone from sheep spleen and from calf thymus on the nucleoprotein phosphorus (NPP) content of cultures grown with and without chick embryo extract*

Test no.	Test substance	Method of sterilization of histone	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
124	Thymus histone + chick E.E.*	L3 candle	6·5 + 30	+0·71
	"	"	1 + 30	+0·92
	Spleen histone + chick E.E.	"	4 + 30	+0·79
	"	"	1 + 30	+0·35
	Thymus histone + chick E.E.	Heat	4 + 30	+0·61
	Spleen histone + chick E.E.	"	5 + 30	+0·77
	Chick E.E. alone	—	30	+0·56
146	Thymus histone	Benzene	11	−0·07
	Spleen histone	"	1·5	−0·05
	Thymus histone + chick E.E.	"	11 + 28	+0·75
	Spleen histone + chick E.E.	"	1·5 + 28	+0·57
	Chick E.E. alone	—	28	+0·49
150	Spleen histone	Benzene	8	−0·07
	Spleen histone + chick E.E.	"	8 + 30	+0·83
	Chick E.E. alone	—	30	+0·61
158	Thymus histone	Benzene	6·5	+0·15
	Thymus histone + chick E.E.	"	6·5 + 30	+0·54
	Chick E.E. alone	—	30	+0·29

* E.E. = embryo extract.

Several methods were used for obtaining sterile solutions of the histones. In the first experiment, histones were dissolved in water, and the solutions sterilized by heating at 100° for 15 min., cooled and treated with an equal volume of double-strength Tyrode solution. Another portion of each histone was dissolved in Tyrode solution and passed through an L3 Chamberland candle. In subsequent experiments the dried histone preparations were sterilized under benzene and dissolved in Tyrode solution. The amount of histone which could be brought into

been made to maintain pure strains over long periods of time.

Both chick and sheep embryo extracts are active in producing rapid increases in NPP, though a comparison of extracts at the same N concentration showed that extracts of chick tissue were superior. For this comparison the 50% sheep extract had to be considerably diluted, and it is possible that comparable concentrations of the active factors in the two extracts are not associated with equal N concentrations. Previous workers have not com-

pared the potencies of extracts of different species on any quantitative basis, though heterologous extracts have often been successfully used (Fischer, 1924; Mottram, 1927; Fischer, 1941*a*; Carrel & Ebeling, 1923; Landsteiner & Parker, 1940; Gey & Gey, 1936). Fischer (1941*a*, 1942*a*, *b*) noted that higher degradation products of homologous proteins were more effective than those of heterologous tissues. Removal of fat from liquid extracts by freezing with ether, besides removing lipid materials which may be inhibitory (cf. Mayer, 1936; Baker & Carrel, 1925), reduces the viscosity of the extracts, which can then be much more easily passed through a filter candle.

It is apparent that sterile embryo extract can be stored at $-30°$ for long periods without losing its activity. At this temperature bacterial action (Reay, private communication) and enzyme action are almost negligible.

Of all the dried preparations made, only those dried from the frozen state were easily soluble. Those dried at room temperature contained much insoluble matter, although the soluble part retained some activity. Treatment with benzene improved the activity, again suggesting the removal of inhibitory lipids, and also provided a convenient method of sterilizing small amounts of powder.

Much of the material present in ethanol precipitates of embryo tissue extracts was also insoluble, though the small amount which did dissolve showed a definite growth-promoting activity. Fischer (1941*b*) found that no fraction of embryo extract was as active as untreated extract, although precipitation with ethanol 'gave a rather good result'. On the other hand, ethanol precipitates prepared in the same way from extracts of adult sheep heart showed very poor activity under the conditions of our experiments. This is contrary to the findings of Doljanski who states that, under his conditions, certain adult organs (including heart) activate the growth of fibroblast colonies *in vitro* to a greater extent than embryonic extract of the same concentration, and that the activity shown by ethanol precipitates is not inferior to that of the native extract (Auerbach & Doljanski, 1944). He does not, however, record any direct comparison between materials prepared from the embryo heart and from the adult heart, although extract of adult fowl heart is stated (Doljanski & Hoffman, 1939) to be more potent than chick embryo extract in increasing the area of tissue cultures. When a comparison is made between ethanol precipitates similarly prepared from adult and embryo sheep hearts, embryo material is found to be much more potent in rapidly increasing the NPP of the cultures.

Comparison of extracts of different embryonic organs confirms the inhibitory effect of liver extracts (cf. Walton, 1914; Heaton, 1926; Brues, Subbarow, Jackson & Aub, 1940). Of the other tissues, cartilage, skin and muscle were all effective. It may be significant that all these contain large amounts of muco- or sulpho-polysaccharides. The high activity of sheep embryo muscle extracts is in contrast to the almost complete absence of activity found by Trowell & Willmer (1939) in extracts of adult fowl muscle. Of the different parts of the chick embryo tested by Fischer (1941*b*) extracts of brain were intermediate in activity between those of the body which were most active and those of the internal organs. The nucleic acid contents of sheep embryo tissues have already been recorded (Davidson & Waymouth, 1944*a*), and there appears to be no relationship between the nucleic acid content (or the nucleotide content) of a tissue and the growth-promoting activity of extracts of that tissue. Although the nucleoprotein fraction prepared from fresh liquid embryo extract is inactive, nucleoproteins prepared by Hammarsten's method (1920) from dried sheep embryo extract have a definite growth-promoting power at low concentrations. These preparations resemble the 'embryonin' prepared from dried beef embryo pulp by Fischer & Astrup (1943), who have stressed the importance of using dried embryonic tissue as the starting material. Drying is reported to render insoluble a large amount of accompanying inactive protein.

According to the hypothesis of Stedman (1944), the amount of basic protein in the nucleus may condition the ability of a cell to divide, so that when sufficient histone is present to combine with all the nuclear nucleic acid, the formation of a nucleic acid-acidic protein (chromosomin) complex with self-reproducing properties is precluded. This hypothesis has been supported (Stedman, Stedman & Pettigrew, 1944) by evidence of the retardation of the growth of tumour material which had been injected as an emulsion with histone into experimental animals. Solutions of histone included in the tissue culture media were found to have no inhibitory effect on nucleoprotein synthesis in the presence of embryo extract, and indeed generally caused a slightly greater rise in NPP than did the extract alone. The maximum concentration of histone which we could obtain in Tyrode solution at pH 7·4 was much less than the 2% present in the emulsion of Stedman *et al.* (1944). It is, moreover, by no means certain that the histone in the form added to the medium was available to the tissue, since the isolated protein differs in physical condition and in chemical association (cf. Macheboeuf, 1944) from the histone *in vivo*. A similar uncertainty must exist about the availability of the isolated nucleoproteins and the fact that the nucleoprotein fraction from dried embryo extract is active again raises the question of the nature of the growth-activating principles of embryo juice.

The recent work of Fischer (1939, 1940, 1941*a*, *b*) and of Fischer & Astrup (1943) suggests that some factor in 'embryonin' acts as a growth-promoting biocatalyst or growth hormone, but whether such a factor is present in the same form in fresh embryo juice is not yet clear. A distinction between nutrient and purely catalytic functions is difficult to draw. The experiments of Fischer (1941*a*) with dialyzed culture media have shown, for example, that embryo extract is ineffective in the absence of the appropriate amino-acids, which are required to maintain the life of the tissue. It is certain that growing cells have higher energy requirements than resting cells, for 'growth is...a process which involves a high rate of metabolism and consumes much energy, but the actual source of that energy is comparatively unimportant' (Willmer, 1935), and it is conceivable that the provision of unnaturally high concentrations of the normal metabolites might itself constitute a primary stimulus to cell division (Calkins, Bullock & Rohdenburg, 1912). Needham (1942), in this connexion, states that the 'growth-promoting factor is probably no more than a right conjunction of nutrient materials and the appropriate capacities for making use of them'. The 'appropriate capacities' may still include specific substances of growth hormone type, but they will certainly depend for their effectiveness on the nutritive requirements of the cells being first fulfilled. Growth catalysts might, moreover, be of two types; those which increase the rate of growth by decreasing the mitotic and/or intermitotic period and those with the true growth-promoting effect of stimulating an increased number of cells to undergo division.

In the presence of the essential nutrients (and as Berrill (1943) points out, the failure of an adult cell to grow *in vivo* is 'clearly not due to lack of nutritive substances or to any deficiency on the part of the cell') proliferation will occur only if the nutrients are available to the cell. According to Berrill, who lays emphasis on the physical as well as the chemical conditions in the tissue, cell division 'is the climax of a complex process of growth and duplication of a multitude of cell components', which must 'occur when cell size reaches a certain critical value', in order to maintain certain volume-surface ratios. The question must still remain open whether one specific tissue component is the limiting factor determining cell division or whether the provision of an optimum environment is itself sufficient stimulus. Nucleoproteins have been considered from this point of view, and it has been suggested that a high concentration of nucleic acid is required for mitosis to take place (Caspersson, 1936; Darlington & La Cour, 1940; Darlington, 1942). Nucleoprotein synthesis in growing tissue cultures may be very rapid and precede cell division by some hours (Willmer, 1942, 1943; Davidson & Waymouth, 1944*d*), and the effectiveness of embryo extract may be due to its capacity to provide both the energy requirements of the tissue and the materials for nucleoprotein synthesis in a readily available form.

SUMMARY

1. The effect of various tissue extracts on the nucleoprotein phosphorus (NPP) content of chick heart fibroblasts growing *in vitro* has been examined. It has been found preferable to express the results as absolute increases in NPP rather than as relative increases.

2. Chick embryo extract has a greater growth-promoting power than sheep embryo extract at the same N concentration.

3. The activity of the extracts is easily impaired by procedures which denature the proteins.

4. Increased activity results from defatting the extracts. Defatted extracts are further improved by drying from the frozen state.

5. Of various procedures adopted for drying embryo extracts, only drying from the frozen state gives satisfactory products.

6. Finely divided powders can be sterilized by standing under benzene or ether, and can then be redissolved aseptically. This procedure obviates the use of filter candles for sterilizing extracts.

7. Sheep embryo extract retains good growth-promoting activity when stored at $-30°$ for 9 months in the sterile state.

8. The growth-promoting activity of chick or sheep embryo extract cannot be attributed to any particular organ or tissue.

9. Ethanol precipitates of embryonic tissue extracts are more active than similar preparations from adult sheep heart.

10. A nucleoprotein fraction prepared from dried sheep embryo extract is effective in low concentrations.

11. Solutions of histone in low concentrations do not impair, but appear to increase, the effect of embryo extract on the NPP of fibroblasts.

12. It is stressed that the normal nutritive requirements of a tissue must be satisfied before the effects of growth stimulants can be assessed, and that these effects may depend on physical as well as purely chemical characteristics of the stimulating materials.

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The Lysine Requirements of the Adult Rat

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Osborne & Mendel (1915), who found that the presence of an adequate amount of lysine in the diet is necessary for growth, also investigated the question of whether or not lysine is needed for maintenance of weight in the adult rat. They observed that a diet in which all the nitrogen was supplied by zein to which tryptophan had been added, and also by 'protein-free' milk, maintained the body weights of their rats for as long as 182 days. These animals showed wasting of muscles, but remained vigorous and began to grow again when lysine was supplied. The conclusion which Osborne & Mendel (1915, 1916) had reached, that lysine was most probably not required for maintenance, has been questioned by later authors. Thus, it is very doubtful, as Osborne & Mendel (1916) themselves acknowledge, whether 'protein-free' milk is really free from lysine. The term 'maintenance' needs to be more clearly defined. As pointed out by Hart, Nelson & Pitz (1918), maintenance in this connexion means that the nitrogen or protein content of the body should be constant over fairly long periods, and constancy of body weight is in itself not sufficient evidence that this has been achieved. Nitrogen balance experiments in rats (Wolf & Corley, 1939) and in dogs (Rose & Rice, 1939) appeared to show that lysine was indispensable for maintenance. On the other hand, Burroughs, Burroughs & Mitchell (1940), using mixtures of essential amino-acids, found that the lack of lysine in the diet of adult rats led to a small or negligible N balance; these authors concluded that lysine is probably not essential for maintenance of N equilibrium in the rat. From experiments in which deaminized casein was fed to volunteers (Albanese, Holt, Brumback, Hayes, Kajdi & Wangerin, 1941) it was deduced that lysine was essential for the maintenance of N equilibrium in humans; these experiments are, however, not conclusive, since deaminized casein is toxic and its deleterious effects are specifically relieved by lysine (Hogan, Powell & Guerant, 1941). In view of the conflict of opinion it was decided to reinvestigate the problem. In earlier experiments (Harris, Neuberger & Sanger, 1943) it was found that young rats kept on diets containing zein and a liver concentrate as the only sources of amino-acid-N did not lose weight, though they did not grow, results which are in agreement with the findings of Osborne & Mendel (1915, 1916) and other workers. According to the most careful analyses (Vickery, 1938; Zittle & Eldred, 1944) zein is free from lysine, but the liver extract supplied about 5·5 mg. N of unknown chemical nature per day. It thus appeared that, if lysine is essential for maintenance, the requirements must be small. It was therefore decided to leave out any supplement of unknown composition and to compare diets, made up of zein, tryptophan and the known crystalline B-vitamins, with a similar diet supplemented with lysine, in their effects on the maintenance of weight in adult rats over long periods.

EXPERIMENTAL

Fully grown male black-hooded and albino rats from our stock were used. Weights were measured twice weekly and food consumption was recorded. The basal diet was similar to the one described (Gillespie, Neuberger & Webster, 1945); it differed in only two respects; the percentage of zein was 15, the starch content of the diet was correspondingly increased; secondly, the amounts of the vitamin B supplements, choline and inositol, were halved. When lysine was added, it was incorporated in the diet; food was given *ad libitum*.

RESULTS

The weight changes recorded in Table 1 for adult rats and also the results reported for adolescent animals (Gillespie *et al.* 1945) on a similar diet show

Table 1. *Changes of body weight of rats receiving basal diets with and without added lysine*

Rat no.	Days on diet	Diet	Body weight (g.) Initial	Final	Average change per week	Daily food consumption (g.)
1	98	Basal	287	218	−4·9	11·5
2	91	,,	290	225	−5·0	11·4
3	56	,,	277	233	−5·5	12·0
4	56	,,	309	273	−4·2	12·2
5	56	,,	237	208	−3·5	11·1
6	50	,,	344	305	−5·6	10·4
7	98	+1% lysine HCl	290	295	—	13·1
8	119	,,	287	289	—	14·2

clearly that a loss of body weight invariably occurs if lysine is absent from the diet. The loss is continuous, as shown in Fig. 1, and the amount of body

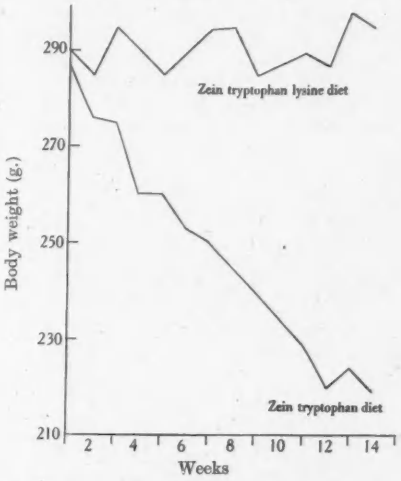

Fig. 1. Weight changes of adult rat on zein diets supplemented with tryptophan with and without added lysine.

substance lost appears to be related to the initial weight. Thus in this series the average weekly loss of weight varied between 1·4 and 1·9 % of the initial body weight with an average figure of 1·75 %, whilst with the adolescent animals of initial weight between 110 and 140 g. an average figure of 1·5 % was found (Gillespie *et al.* 1945). If lysine is added to the diet, no loss of weight is observed; in some cases slow growth, even in adult animals, has been found. It must be concluded, therefore, that, at least with the diet used in these experiments, the presence of lysine in the food is essential for the maintenance of body weight in adolescent and adult rats.

The lysine-deficient animals appeared to be quite healthy, even after having been on this diet for nearly 100 days. Their fur was quite smooth and there was no marked wasting of muscles, but the subcutaneous fat was scanty compared with that of the control animals. They also showed marked hypoproteinaemia. It should be noted that these observations were made before those on adolescent rats (Gillespie *et al.* 1945) and as the occurrence of anaemia was not then under consideration no haemoglobin estimations were made. Histological examination of liver, kidney, spleen, testis and bone-marrow, kindly carried out by Dr Glynn, showed no pathological changes. It has been claimed (Albanese, Holt, Frankston, Kajdi, Brumback & Wangerin, 1943) that lysine deficiency in humans caused increased excretion of non-ketonic organic acids in the urine. We have been unable to detect such an increase in lysine-deficient adult rats; this may be due to a species difference. It is also possible that the increase of urinary organic acids observed by the American authors was not due to the lack of lysine in the diet, but was caused by some factor present in the deaminized casein. It may be concluded, therefore, that the absence of lysine in the diet of the adult rat causes no specific lesions.

In the next experiment increasing amounts of lysine were added to the diet and the effects on body weight observed. It can be seen (Table 2) that an intake of up to 10 mg. of lysine hydrochloride per day causes no significant change in the loss of weight. A dose of about 14 mg. reduces the loss of body weight by about one-half, whilst an intake of about 20 mg. of the hydrochloride ensures on the average a maintenance of weight. Higher doses of lysine hydrochloride, i.e. 25 mg. or more, led to an increase of weight even in these rather adult rats. 20 mg. of lysine hydrochloride, or 16 mg. of lysine itself, may be considered the minimal dose for maintenance in a rat of about 300–350 g. body weight.

DISCUSSION

The finding that the presence of lysine in the diet is essential for maintenance of the body weight of the adult rat is in accordance with results obtained in

Table 2. *Changes of body weight of adult rats receiving basal diets with varying amounts of lysine*

Rat no.	Days on diet	Daily lysine HCl (mg.)		Body weight (g.)			Daily food consumption (g.)
		Added	Eaten	Initial	Final	Average change per week	
18	26	15·0	13·3	240	228	−3·0	12·8
19	26	15·0	13·6	253	245	−2·0	13·1
20	26	15·0	13·8	252	241	−2·7	13·3
25	27	20·0	17·1	305	296	−2·2	13·4
26	27	20·0	18·2	380	380	0	14·2
27	27	20·0	18·8	324	321	−0·7	14·7
30	26	25·0	20·6	310	305	−1·0	14·4
31	26	25·0	24·0	358	380	+5·5	17·4
32	26	25·0	22·6	307	324	+4·2	15·8

other species. The contrary findings of Osborne & Mendel (1915, 1916) may be ascribed to the presence of small amounts of lysine in their 'protein-free' milk. The loss of body weight in lysine deficiency is slow and the over-all loss of N is small. This may explain why Burroughs *et al.* (1940) were unable to establish with certainty a negative N balance in lysine-deficient rats. We have obtained similar equivocal results with the balance method, which is not sufficiently sensitive to detect small differences between intake and excretion. Another point has also to be considered. In the experiments reported here diets were used that did not contain biotin, folic acid and other less well-defined growth factors. If bacterial synthesis of amino-acids in the intestine is of any importance to the lysine supply of the rat, it is possible that the omission of these growth factors from the diet may affect adversely the microbiological synthesis in the gut, and may thus modify the dietary requirements for lysine. This point must, however, await further investigation, when all these factors have become available in a pure form.

There is a considerable disparity between the maintenance requirements of the adult rat for lysine and the requirements of the young animal for optimal growth. Thus the minimal value for maintenance obtained from the present work is about 16 mg. a day, which is equivalent to about 0·1% of the total food. Rose (1938) states that the young animal requires about 1% of lysine in its food for optimal growth. This estimate, which agrees with our (unpublished) observations, means that a young rat needs about 100 mg. of lysine a day for optimal growth; the ratio between growth requirement of the young animal and the maintenance requirement of the adult would therefore be, in absolute amounts, about 6:1. This ratio would become considerably greater—about 20 or 25:1—if the comparison were made on the basis of body surface. Whatever the correct basis for this calculation, it is clear that the maintenance requirement for lysine is small compared with the growth requirement. There are no comparable figures for other amino-acids, but the results of Burroughs *et al.* (1940) indicate that dietary deficiencies of threonine, isoleucine and methionine produce very abrupt and marked nitrogen losses from the body, whilst the omission of other essential amino-acids like lysine and histidine causes no such negative balance as could be established with certainty. Rubner has called the loss of nitrogen which occurs through irreversible oxidation of amino-acids the 'wear and tear' quota, a quota which has to be replaced if the body protein is to be maintained. It appears now that this 'wear and tear' quota is a factor characteristic not so much for protein as a whole, but for its constituent amino-acids and will differ in value from one amino-acid to another. A low 'wear and tear' quota for lysine such as is postulated here, is in accord with the relative metabolic inertia shown by lysine towards enzymes and tissue slices (Neuberger & Sanger, 1944).

SUMMARY

1. It has been shown that the presence of lysine in the diet is necessary for the maintenance of body weight in the adult rat.

2. The amount of lysine required for maintenance has been determined and found to be very much smaller than the amount needed for optimal growth.

3. It is suggested that the relative proportions of essential amino-acids required for growth and for maintenance respectively may vary considerably for different amino-acids.

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Further Studies on Lysine Deficiency in Rats

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In an earlier paper (Harris, Neuberger & Sanger, 1943) the effects in young rats of a diet containing only small amounts of lysine were studied. The main symptoms of this deficiency were: cessation of growth without any marked loss of weight, wasting of muscular tissue, retarded bone formation, and reduced mitotic figures in the testes. Serum protein values were well below normal and there was also a reduction in the number of red cells and the amount of haemoglobin per unit volume of blood. The interpretation of the haematological data was rather difficult, because both haemoglobin and the number of red cells increase considerably from birth to pubescence, when the adult values are reached (Metcoff & Favour, 1944; Albanese, Holt, Kajdi & Frankston, 1943). A similar increase is also found in the protein content of plasma and it was therefore possible that the low values found in these lysine-deficient rats might have been due to a retention of infantile characteristics caused by the cessation of growth and were not indicative of a true anaemia or hypoproteinaemia. The experiments to be reported in this paper were, therefore, undertaken with older animals in which the number of red cells and the content both of haemoglobin and plasma proteins approached constancy. The absence of lysine from the diet was also more rigorously secured than in the earlier experiments.

The nutritional requirements for a particular amino-acid are usually measured by recording the changes of body weight of an experimental animal on diets in which the intake of that amino-acid is varied. It is obvious that changes of body weight will give only a very crude idea about variations of the protein content of cells and of that of the body as a whole. Thus it appeared possible that, since a diet deficient in an essential amino-acid produces a hypoproteinaemia, this might be associated with an oedema of the tissues. The increased water content of the body would then largely mask a real loss of weight, i.e. a loss of body substance. Moreover, as pointed out by Mitchell & Carman (1926), a relative increase of fat produced by a diet will also either mask or exaggerate a net gain or loss of body protein, if body weight is used as a criterion. It seemed, therefore, important to estimate both the water and the nitrogen content of the tissues; such measurements appeared to be particularly important in the case of the liver, which occupies such a central position in protein metabolism.

In the earlier paper the question was discussed how far the observed changes in lysine deficiency were due directly to a lack of this amino-acid in the diet and how far they were primarily the result of a decreased food intake brought about by the amino-acid deficiency. We have therefore compared, in the present work, one group of rats on a lysine-deficient diet given food *ad libitum* with a second group receiving the same diet supplemented with lysine, the food also being given *ad libitum*; a third group of animals received the stock laboratory diet which was so restricted in amount as to produce a decrease in weight similar to that observed in the experimental group. This represents a modification of the paired-feeding method, a modification in which the body weights and not the food consumptions are equalized. Any differences found between the first and third groups could be considered to be caused specifically by the lack of lysine, and not by a restriction of food intake.

EXPERIMENTAL

Three litters of albino rats of the Wistar strain, with weights varying between 110 and 140 g., were obtained from Glaxo Laboratories. The animals were distributed uniformly with respect to sex among the different groups. They were kept in screened cages to exclude coprophagy. Weights were recorded twice weekly, and food consumption was also measured.

Diet. The basal diet (ZT) was as follows: zein 18%, maize starch 60·8%, arachis oil 14·4%, cod-liver oil 1·6%, *l*(−)-tryptophan 0·2% and salt mixture (U.S. Pharmacopoeia II, no. 2) 5%. In the control diet (ZTL) 0·5% of the starch in the basal diet was replaced by the same amount of *l*-lysine hydrochloride which had been prepared in this laboratory and had the correct composition and optical rotation. The water-soluble vitamins were given as a separate supplement in the following daily doses: aneurin 60 μg., pyridoxin 60 μg., riboflavin 100 μg., calcium *d*-pantothenate 200 μg., inositol 2 mg., nicotinamide 2 mg., choline chloride 6 mg. No additional concentrates were given to provide folic acid or biotin. The stock diet (SR) which was given in restricted amounts to a third group of rats had the following composition: rolled or ground oats 30%, wholemeal flour 35%, dried yeast 6%, dried skim milk 15%, meat and bone meal 10%, cod-liver oil 2% and salt mixture 2%.

Haemoglobin and red cell number. These were estimated at the beginning and end of the experiment and also at infrequent intervals during the experimental period. Measurements during the life of the rats were reduced to a minimum in order to avoid the loss of protein resulting from the loss of blood. Free-running blood was obtained by incision of the tail. All haemoglobin estimations were carried out by the same observer, using all necessary precautions to produce good matching and reproducible readings. A standard checked by the National Physical Laboratory was used and it was assumed that a reading of 100% corresponded to a haemoglobin content of 14·8 g./100 ml. of blood (King, Gilchrist & Matheson, 1944).

Protein, fat and water content. The three litters were killed by inhalation of carbon monoxide after 63, 75 and 85 days respectively. Blood was removed from the heart and used for the estimation of plasma proteins by the method of Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald (1943). The application of this method to rat plasma may involve a systematic error, as pointed out by Metcoff & Favour (1944) which, however, is likely to be constant. Comparison with results obtained by the Kjeldahl method is difficult, since the conversion factor for rat plasma protein is not well established. The liver was taken out, rinsed under the tap, and the excess water then removed by wiping on a towel. The liver was then weighed, cut into small slices, reweighed and the dry weight then estimated after drying to constant weight in a vacuum oven at 100°. Fat was estimated by extracting the dry liver powder with ether in a Soxhlet apparatus for 8 hr. The term 'fat' used in this paper denotes material which is extracted by ether under these conditions. The defatted residue was then used for the estimation of N by the Kjeldahl method. The 'protein' content was calculated by multiplying the N content by 6·25. It is realized that this factor is arbitrary and also that the true protein-N content is smaller than the total N. It was found, however, that the non-protein-N is small, about 6–8% of the total N, both in liver and carcass, and that the relative proportion of non-protein to protein-N is not significantly altered by the diets used.

Skin, tail, head and intestinal organs were then removed, and the remaining carcass then cut up and dried as described for the liver. Fat and protein contents were then estimated as described above.

RESULTS

Body weights. Table 1 shows that the rate of growth of rats receiving a zein diet supplemented with tryptophan and lysine (ZTL) varies considerably even in rats of the same litter. The average increase of weight, which amounts to about 8 g./week, is similar to the value found for younger animals (Harris *et al.* 1943) at the same protein level in the diet. The food consumption runs parallel to the rate of growth and it thus appears that the variation in growth rates of the different rats is caused by differences in appetite. The rats receiving a diet which does not contain lysine (ZT) lose weight at varying rates; the average weekly loss of weight as percentage of the initial weight is about 1·5%, which compares well with a value of 1·8% found for

Table 1. *Changes of weight, red cell number, haemoglobin and plasma protein contents of rats receiving zein diets with (ZTL) and without (ZT) added lysine and of rats on a stock diet with restricted food intake (SR)*

(The letters A, B and C in column 1 refer to the three litters used.)

Rat no.	Body weight (g.) Initial	Final	Days on diet	Diet	Blood haemoglobin (g./100 ml.) Initial	Final	Red cells (millions/cu.mm.) Initial	Final	Plasma protein (%) Final	Average daily food consumption (g.)
A 1 ♀	106	170	75	ZTL	13·6	14·2	7·98	8·48	7·03	12·3
A 6 ♂	151	279	75	,,	12·7	15·1	7·82	8·96	7·03	17·5
A 8 ♂	137	224	75	,,	13·0	16·3	7·72	9·67	5·95	18·6
B 9 ♂	140	256	63	,,	13·8	14·2	7·48	8·59	7·15	16·7
B 13 ♀	113	167	63	,,	14·5	15·1	7·40	8·96	6·36	11·6
C 17 ♂	119	151	85	,,	13·0	14·5	8·10	8·00	6·04	9·6
			Mean value		13·5	14·9	7·75	8·76	6·58	12·2
A 2 ♀	111	102	75	ZT	13·6	13·0	8·92	7·12	5·49	9·2
A 7 ♂	140	115	75	,,	13·0	10·7	7·76	6·56	5·15	9·4
B 11 ♂	136	113	63	,,	13·0	10·3	6·66	7·20	4·75	7·8
B 12 ♀	110	97	63	,,	13·3	9·5	6·84	7·12	5·10	9·3
B 15 ♀	110	88	63	,,	14·2	10·3	7·36	5·46	5·95	8·1
C 16 ♂	111	88	85	,,	13·0	11·8	6·86	7·64	5·80	7·1
C 18 ♀	107	92	85	,,	13·3	12·1	8·00	8·00	5·01	6·0
			Mean value		13·4	11·1	7·48	7·10	5·18	8·1
A 3 ♀	109	111	75	SR	13·0	16·0	9·24	9·92	6·96	10·3
A 4 ♂	124	103	75	,,	13·0	14·5	7·72	8·63	5·90	10·3
A 5 ♂	139	103	75	,,	13·3	16·0	6·56	9·84	5·70	10·3
B 10 ♂	130	115	63	,,	12·7	12·1	7·52	8·60	5·82	10·3
B 14 ♀	130	117	63	,,	13·0	15·7	7·48	9·44	6·15	10·3
C 19 ♀	101	91	85	,,	13·6	15·7	7·82	8·76	6·31	10·3
C 20 ♀	113	113	85	,,	12·7	15·4	6·90	7·60	5·76	10·3
			Mean value		13·1	15·1	7·60	8·96	6·08	10·3

heavier animals (Neuberger & Webster, 1945). The restriction of food consumption for the animals on the stock diet (SR) to a uniform level has not succeeded in producing identical losses of body weight; the average decrease of weight is slightly less than in the ZT group. Histological examination of the liver, kidneys, thyroid, bone-marrow and testis was kindly carried out by Dr L. E. Glynn. There were no consistent histological changes associated with the different dietary groups. The ZT group showed a tendency to testicular atrophy with reduced spermatogenesis; but these changes occurred only in some animals and similar changes, though to a lesser extent, occurred in some animals on the reduced stock diet.

Haemoglobin and red cells. In spite of the appreciable variations which occurred within one group it can be seen from Table 1 that the average initial figures both for haemoglobin and red cells are similar for the three groups. Both haemoglobin content and cell number rise during the experiment in groups ZTL and SR to the full normal adult values, though the rise of the two sets of figures is not parallel for individual rats. Restriction of food intake on an otherwise adequate diet does not therefore lead to anaemia, although it produces a loss of body weight. The lysine-deficient animals (group ZT), however, show a marked fall both in haemoglobin content and number of red cells, and the final average values for haemoglobin are about 30% below those of the two control groups, whilst the average difference for cell number is about 20%. The decrease in haemoglobin content is slightly greater than the drop in cell number. The ratio of haemoglobin content, in g./100 ml., and the number of red cells, in millions/cu.mm., can be used as a measure of the 'colour index', i.e. for the average haemoglobin content of the individual erythrocyte. This ratio tends to be lower in lysine-deficient rats, although individual variations are considerable. It may, therefore, be said that this anaemia is probably of the hypochromic type. Haemoglobin estimations carried out during the experimental period indicated that this anaemia does not develop until the animals have been on the lysine-deficient diet for at least 40 days. Similar observations have been made on the anaemia produced in rats by dietary deficiency of tryptophan (Albanese *et al.* 1943).

Plasma proteins. The plasma protein values also vary considerably in each group. In the ZT group the figures vary between 4·75 and 5·95% with a mean value of 5·18%; the variations in the two controls are 5·7–7·15% and 5·7–7·0%, with mean values of 6·4 and 6·1% respectively. If litter-mates are compared the differences are even more significant. There is therefore an average reduction of plasma proteins in lysine-deficient animals of about 15–20% as compared with the two control groups. The hypoproteinaemia is less severe than that found in younger animals (Harris *et al.* 1943).

Water content of the carcass and the liver. The part of the carcass used for these estimations represents only about 50% of the total body weight; hair and skin have also been examined in a number of rats; but since no additional information of any interest has been obtained, these results are not reported here. The water contents of the carcasses for all three groups are about equal and within normal limits (Table 2). The water content of the livers of the lysine-deficient animals (ZT) tends to be higher than in the two control groups (Table 3). In view of the variations found, it is, however, doubtful whether the difference is of statistical significance. If there is any increased water retention in the livers of ZT animals as compared with the controls, it is only very small.

Table 2. *Water, fat and protein contents of carcasses of rats receiving normal (ZTL), lysine-deficient (ZT) diets and stock diets with restricted food intake (SR)*

Rat no.	Diet	Carcass: Weight (g.)	Carcass: Fat content (%)	Carcass: Water content (g./100 g. fat-free carcass)	Carcass: Protein content (g./100 g. fat-free carcass)
A 1 ♀	ZTL	77·8	18·0	72·1	21·1
A 6 ♂	„	126·3	19·5	72·5	20·5
A 8 ♂	„	98·5	16·0	72·5	21·1
B 9 ♂	„	117·1	17·5	73·2	20·9
B 13 ♀	„	78·4	12·5	73·9	19·7
	Mean value		16·7	73·0	20·7
A 2 ♀	ZT	47·3	11·7	73·6	19·7
A 7 ♂	„	54·2	8·6	73·7	19·6
B 11 ♂	„	42·9	8·3	73·8	20·0
B 12 ♀	„	42·9	12·4	73·3	20·3
B 15 ♀	„	36·1	8·1	73·7	19·8
	Mean value		9·8	73·6	19·9
A 3 ♀	SR	53·7	8·4	72·6	20·3
A 4 ♂	„	46·6	1·3	71·5	20·1
A 5 ♂	„	46·2	1·3	72·8	19·4
B 10 ♂	„	53·5	2·9	73·1	20·0
B 14 ♀	„	52·5	7·2	73·0	20·0
	Mean value		4·3	72·6	20·0

Fat content of the carcass and the liver. The fat content both of the carcass and of the liver differs considerably for the different groups. The animals on the restricted stock diet have very low fat contents; the fat deposits have practically disappeared. The fat content of the livers of the rats of the ZTL is fairly high, particularly if compared with that of the lysine-deficient group (ZT). The high fat content found in some of the livers of the ZTL group was rather unexpected and the design of the experiment does not permit us to establish with certainty the reason for the occurrence of these fatty livers. All

Table 3. *Water, fat and protein content of livers of rats receiving normal (ZTL), lysine-deficient (ZT) diets and of rats on stock diet with restricted food intake (SR)*

		Liver					
						Protein content	
Rat no.	Diet	Weight (g.)	Fat content (%)	Fat-free tissue (g./100 g. body wt.	Water content (g./100 g. fat-free tissue)	g./100 g. fat-free tissue	g./100 g. body wt.
A 1 ♀	ZTL	5·331	10·2	2·81	73·1	20·3	0·573
A 6 ♂	,,	9·608	6·9	3·02	75·0	20·3	0·615
A 8 ♂	,,	8·781	3·5	3·41	76·3	19·1	0·672
B 9 ♂	,,	9·709	16·4	3·06	73·7	21·2	0·650
B 13 ♀	,,	7·076	17·5	3·50	75·2	19·2	0·672
C 17 ♂	,,	4·854	5·5	3·04	69·9	19·8	0·601
	Mean value		10·0	3·14	73·9	20·2	0·631
A 2 ♀	ZT	3·372	6·1	3·11	74·3	16·9	0·527
A 7 ♂	,,	3·798	5·0	3·15	75·0	15·9	0·501
B 11 ♂	,,	4·338	5·7	3·62	75·1	15·8	0·571
B 12 ♀	,,	3·419	4·0	3·38	74·0	17·6	0·584
B 15 ♀	,,	2·765	4·2	3·01	75·9	17·9	0·530
C 16 ♂	,,	2·876	3·8	3·15	74·7	16·1	0·506
C 18 ♀	,,	2·421	5·2	2·50	73·3	20·1	0·503
	Mean value		4·9	3·28	74·8	17·2	0·533
A 3 ♀	SR	3·172	3·2	2·79	72·3	21·3	0·596
A 4 ♂	,,	3·040	3·3	2·86	73·9	20·7	0·593
A 5 ♂	,,	2·908	2·1	2·77	72·7	21·1	0·585
B 10 ♂	,,	3·462	1·0	2·97	73·0	21·0	0·623
B 14 ♀	,,	3·125	2·3	2·61	73·3	23·1	0·613
C 19 ♀	,,	2·778	1·8	3·01	70·7	20·4	0·618
C 20 ♀	,,	3·184	2·5	2·76	13·1	23·2	0·640
	Mean value		2·3	2·82	72·7	21·5	0·610

the rats received choline, though possibly a sub-optimal amount. Zein is known to have only a weak lipotropic effect (Channon, Loach, Loizides, Manifold & Soliman, 1938) and this, together with the low choline and rather high aneurin content of the diet and the possible absence of some of the factors of the vitamin-B group, may be responsible for the high fat content found in the livers and also in the carcasses of the rats receiving the ZTL diet. The reduction in the fat content of the body and the liver which is found on leaving out lysine (ZT group) may be explained by the fact that these animals do not grow at all, and the methionine which is present in a concentration of 2·35% in zein (Baernstein, 1932) is available for choline synthesis. The suggestion that increased growth accentuates the effects of a choline deficiency by rendering less methionine available for choline synthesis, and also by raising the demand for choline, has been put forward by several workers (Griffith & Mulford, 1941; Treadwell, Tidwell & Gast, 1944). On the other hand, Tucker & Eckstein (1938) have found that fatty livers produced by a gliadin diet are not affected by the addition of lysine.

Protein content of the liver and carcass. The protein content of the carcass is approximately the same for the three groups as shown in Table 2. The liver protein contents, however, differ considerably for the different diets (Table 3). In the SR group the weight of the liver relative to total body weight is reduced; but this is compensated by an increased protein content of the liver in such a way that the amount of liver protein/100 g. body weight is nearly the same as in the ZTL group. It follows, therefore, that restriction of food intake as such does not lead to a reduction of liver protein. The ZTL group shows an increase of liver weight relative to body weight, but this is accounted for by an increased fat content, and if allowance is made for this the relative liver weights are almost the same for the ZT and ZTL groups (Table 3, column 5). The protein content of the liver of the lysine-deficient group is considerably reduced as compared with the two control groups. The reduction is equally great, whether the comparison is carried out on the basis of the amount of liver relative to the dry weight of the liver, or relative to the fresh weight, or on the basis of the ratio of liver protein to body weight. The conclusion can therefore be drawn that lysine deficiency produces a reduction in liver protein which is not due to decreased food intake, as comparison with the SR group clearly demonstrates. Since the content of solids is nearly the same in the livers of all three groups, it follows that the livers of lysine-deficient

animals contain a higher proportion of non-protein matter not extractable by ether, presumably glycogen.

DISCUSSION

The main symptoms produced by lysine deficiency in rats of an initial weight of 100–120 g. are: moderate loss of body weight, a fairly mild anaemia with a slightly greater average reduction in haemoglobin than in cell number, a hypoproteinaemia and a decrease of liver protein. It has been shown that none of these symptoms, apart from loss of weight, is produced by a simple restriction of food intake on a qualitatively adequate diet. The anaemia is mild, compared with an iron-deficient anaemia, and may be due to an inhibition of haemoglobin formation or to an interference with the formation of the structural unit, the red cell. The lysine content of rat haemoglobin is not known, but by analogy with other species it may be assumed that the haemoglobin of the rat contains about 9–10% of lysine, i.e. more than most other proteins in the body. This and the hypochromic character of the anaemia favour slightly the hypothesis that it is the synthesis of haemoglobin which is disturbed. Albanese *et al.* (1943) have recently shown that tryptophan-deficiency produces an anaemia in rats similar to the one described here and it is highly probable that these anaemias are not specific for deficiencies in particular amino-acids, but are the result of an inhibition of formation of protein as a whole. Such an interpretation is supported by the observations of Weech, Wollstein & Goettsch (1937) on dogs. These authors found that a diet very low in protein produces a progressive fall in total circulating haemoglobin. Measurements of haemoglobin concentration per unit volume of blood obscured this decline, since the plasma volume fell more rapidly at first than total haemoglobin. If lysine deficiency in rats also produces such a reduction of plasma volume, the anaemia would be more severe than our figures indicate. This interpretation, which assumes that prolonged deficiency of lysine affects the production of globin, is also in agreement with the work of Hahn & Whipple (1939), who found that the output of new haemoglobin in anaemia due to loss of blood can be kept at low levels by limitation of dietary protein. The results of Pearson, Elvehjem & Hart (1937), who noted that recovery from an iron-deficient anaemia is not greatly influenced by the quality of the protein given during the recovery period, are not in conflict with this conception. In the latter case the protein stores are filled and the limiting factor for growth and haemoglobin formation is the iron intake. If now iron is given, the formation of haemoglobin, which takes precedence over that of plasma proteins, for example (Robscheit-Robbins, Madden, Rowe, Turner & Whipple, 1940), proceeds at the expense of the protein stores of the body. Amino-acid deficiency will therefore only affect the synthesis of haemoglobin when the protein reserves have considerably decreased. Protein has not been much discussed as a causative agent of experimental or clinical anaemia. Recently, however, Orten & Orten (1943) produced a mild, hypochromic anaemia in rats by feeding a diet low in protein but adequate in other respects, and Bethell (1936) reported that the haemoglobin content of the blood of pregnant anaemic women can be raised by an increased protein intake. It appears very probable, therefore, that a deficiency in total protein or particular amino-acids may at least be a contributory factor in the anaemia found in cases of malnutrition.

The reduction of the protein content of the liver and the associated hypoproteinaemia contrast strongly with the apparently normal nitrogen content of the carcass. A progressive fall in serum proteins and a decrease in the protein content and a slight increase in the water content of the liver were also found in dogs fed a diet low in proteins (Elman & Heifetz, 1941). It appears that the liver is particularly sensitive to changes in the amino-acid composition of the diet. This quick reaction of the level of liver protein to dietary changes has already been described by Addis, Poo & Lew (1936); these authors found that the loss of protein resulting from starvation is more pronounced in the liver than in other organs; it occurs at an earlier stage. In a later paper (Addis, Lee, Lew & Poo, 1940) it was shown that the protein content of the liver, kidney and plasma varies considerably with the level of protein in the diet. The effect of changes of dietary protein on the composition of the liver has also recently been examined by Kosterlitz (1944), who found a decrease of liver protein if rats were kept either on protein-free diets or on rations deficient in essential amino-acids like tryptophan. From a simultaneous analysis of the nucleic acid and phospholipid content of the liver the conclusion was drawn that the protein lost is derived from the cytoplasm and that a certain fraction of the cytoplasmic protein is very labile metabolically. The conception of a labile protein store in the liver has also been used to interpret the many observations made by Whipple and his collaborators on the regeneration of plasma protein and haemoglobin after loss of blood or plasma (Madden & Whipple, 1940). Lysine deficiency in the diet apparently reduces the labile protein store in the liver in the same way as a general reduction of protein intake, without affecting greatly the protein content of the body as a whole. The hypoproteinaemia found is best explained by assuming that the liver, and possibly this labile cytoplasmic fraction, is closely concerned with plasma protein formation.

SUMMARY

1. Lysine deficiency in rats of an initial weight of 110–140 g. produces a mild anaemia with a slightly greater reduction in haemoglobin than in red cells. It is suggested that protein deficiency may be a contributory factor in nutritional anaemia.

2. There is also a loss of protein from the liver and a hypoproteinaemia, whilst the body protein content seems to be unchanged. The importance of the liver for protein metabolism and its possible role in connexion with the synthesis of serum proteins is discussed.

3. None of these symptoms, apart from a loss of body weight, is observed if an adequate diet is restricted in amount to produce weight changes similar to those observed in lysine-deficient animals. It is suggested that the clinical picture is produced by a general interference with protein synthesis and does not represent a specific lesion.

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The Pigments of Sponges

1. THE LIPID PIGMENTS OF THE SPONGE *HYMENIACIDON SANGUINEUM* (GRANT)

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The classification of carotenoid pigments into those present in plants, exhibiting the normal triplet spectrum, and those of animal origin, such as astacene, glycymerin and echinenone, with a single-banded spectrum, would not appear to be correct. Recently Heilbron, Jackson & Jones (1935) and Lederer (1938) have isolated animal xanthophylls possessing three absorption bands, e.g. pectenoxanthin, $C_{40}H_{54}O_3$, pentaxanthin, $C_{40}H_{56}O_5$, and sulcatoxanthin, $C_{40}H_{52}O_8$. It would seem, however, that no corresponding animal hydrocarbons have so far been identified. Lederer (1938) has made a study of the carotenoid pigments of some fourteen species of invertebrates and has recorded his failure, save in two cases, to isolate any plant xanthophylls, a striking fact when it is remembered that the food of marine animals may include seaweed containing the xanthophylls fucoxanthin, taraxanthin and lutein. In view of this interesting observation of Lederer and in order to obtain further information with regard to the pigments of invertebrates a study has been made of the colouring matters of certain

Porifera and the present communication deals with the pigments of the sponge *Hymeniacidon Sanguineum* (Grant).

The carotenoid nature of the pigments of sponges was established by Krukenberg (1882), MacMunn (1888) and Lönnberg (1931). The former refers to their carotenoid-like and rhodophane-like pigments, the latter (probably astacene) possessing a single absorption band. These investigators observed the presence of other pigments with two or three bands resembling the carotenes or xanthophylls. Bergmann & Johnson (1933) examined the sponge *Microciona prolifera* and concluded that carotene was present but did not isolate it. More recently Karrer & Solmssen (1935) isolated the crustacean pigment, astacene, from the sponge *Axinella cristagalli* and suggested that it was also present in *Suberites domuncula*. This latter statement is at variance with that of Lederer (1938), who found that the pigments of this sponge were entirely epiphasic, even after saponification, and therefore did not include astacene. Lederer (1938) has isolated β-carotene from the sponge *Ficulina ficus*.

The sponge *Hymeniacidon Sanguineum*, used in this investigation, was obtained from the Marine Biological Station, Lough Ine, Co. Cork. The presence of large amounts of phosphatides and sterols complicated and rendered difficult the isolation of the colouring matter, a fact which might explain the comparative failure of workers in this field. After dehydration of the sponge with acetone the pigments were extracted with ether and the petroleum-soluble unsaponifiable matter partitioned with 90% methanol. The colouring matter was entirely epiphasic. Neither at this nor any subsequent stage was the presence of astacene observed. On chromatographic adsorption on alumina a number of ill-defined coloured zones was formed, the uppermost and main zone being dark red. By development of the chromatogram with mixtures of benzene light petroleum of gradually increasing eluting power it was found possible to wash through the lower zones of the chromatogram. With the use of the spectroscope as a control a fairly sharp separation into two fractions, spectroscopically distinct, was effected. From the first of these fractions α-carotene was isolated in crystalline form. The second fraction, on further purification, yielded β-carotene. From the upper deep red zone of the original chromatogram, on development with benzene, a well-defined violet zone separated and a lower orange-red zone was completely washed through. From the latter was isolated a pigment agreeing in melting-point and absorption maxima with γ-carotene. The pigment from the violet zone, when separated, and rechromatographed twice on aluminium oxide, showed an intense absorption band at 488mμ in carbon disulphide and melted at 192–193°. It would appear to be identical with echinenone, the first animal-provitamin A isolated by Lederer (1935) from the sea-urchin *Strongylocentrotus lividus*. The residual upper zone of the original chromatogram consisted of oxidized pigments or their decomposition products, showing only indefinite absorption spectra. It was not found possible to isolate any definite substance therefrom.

The demonstration of the presence of echinenone in a sponge provides a chemical link between the otherwise unrelated Porifera and Echinodermata. It would appear that this is the first occasion on which γ-carotene has been isolated from invertebrates.

An interesting speculation can be made from the occurrence of astacene in Karrer's deep-water sponge in contrast to the isolation of less oxygenated, non-acidic pigments in the present instance from a shallow-water sponge. The difference may be connected with the penetration of light which may give the pigments a different phytochemical significance in each case. Of interest in this connexion is the influence of light on the pigmentation of the prawn, *Palaemonetes vulgaris* (Brown, 1934). In water, with a white background, the red pigment (astacene) of this animal and its blue chromoprotein are rapidly destroyed in the light. This carotenoid is formed, however, when the background is black.

EXPERIMENTAL

The red sponge *Hymeniacidon Sanguineum* (1 kg. dry weight) was collected in March. After freeing from mussels and algae some of the water was expressed by hand. The sponge was immediately chopped into small pieces, covered with pure acetone, and the whole allowed to remain overnight. The solvent, on being filtered off, was only faintly coloured and was neglected. On repeating once more the above operation, the acetone filtrate assumed a light yellow colour. The dry sponge was now covered with ether (peroxide-free) and the whole left overnight in a dark place. After filtering, the extraction with ether was repeated three times, the final ether extract being only faintly coloured. The united deep-red ether extracts (6 l.) were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure, in an atmosphere of CO_2, the temperature being kept below 30°. The reddish butter-like residue was next saponified by heating at 40° for 3 hr. with 10% methanolic potash, in an atmosphere of nitrogen.

To test for the presence of astacene a small portion of this solution was withdrawn and covered with a layer of light petroleum. On acidification by addition of dilute acetic acid no separation of solid at the interface was observed. The main portion was diluted with water and extracted three times with light petroleum (b.p. 60–80°). The alkali layer was coloured olive-green due, probably, to the presence of chlorophyll and its decomposition products. The deep-red light-petroleum layer was washed with water until free from alkali and methanol and dried with anhydrous Na_2SO_4. As it was found necessary to remove accompanying sterols at this stage, the light-petroleum extract

was concentrated somewhat and placed in the ice-chest overnight. The bulky crystalline mass which separated was filtered off and the filtrate partitioned with 90% methanol. In this way a red epiphasic and a faint yellow hypophasic layer were obtained.

Light-petroleum phase. The light-petroleum layer (1 l.) was freed from methanol by repeated washing with water, dried with anhydrous Na_2SO_4 and adsorbed, in two portions, on two columns (20 × 5 cm.) of aluminium oxide (Merck). The chromatogram consisted of a series of ill-defined zones: (1) deep-red (2 cm.), (2) orange-red, (3) orange-yellow. On development with a 2% solution of benzene in light petroleum an orange-yellow filtrate was obtained and this was collected until a distinct change in its spectral readings was observed (filtrate 1). At this stage further development was continued with light petroleum containing 10% of benzene, when a second orange-coloured filtrate was collected. Here again development with this mixed solvent was continued until a change in absorption spectrum took place (filtrate 2). The upper zone of the chromatogram, which was still deep red, had now increased in depth to 3 cm. On further development with benzene a violet zone gradually made its appearance and a lower reddish band moved into the filtrate (filtrate 3). Washing with benzene was continued until the violet zone had become well defined. The violet zone when separated, eluted, and rechromatographed twice on aluminium oxide furnished a small uniform violet and a lower orange-red zone. Elution of the violet zone and repeated crystallization of the eluate from benzene-methanol furnished a pigment consisting of bluish violet needles melting at 192–193° and showing one broad band at 488 mμ in carbon disulphide. It would appear to be identical with echinenone, first isolated by Lederer (1935), although the latter states that echinenone exhibits, in addition to the broad band at 488 mμ, two very faint bands at 520 and 450 mμ in carbon disulphide.

Filtrate 1. This was evaporated to dryness *in vacuo*, the residue dissolved in light petroleum (60–80°) and the solution shaken with a small quantity of fuller's earth (Kuhn & Brockmann, 1931). The filtrate, on concentration to small bulk, deposited a pigment which on recrystallization from the same solvent was obtained as violet-coloured prisms melting at 182–184°, with absorption maxima in carbon disulphide at 511, 478, 448 mμ and in light petroleum at 478, 448 mμ, in close agreement with the recorded data for α-carotene.

Filtrate 2. This was brought to dryness, the residue dissolved in light petroleum and chromatographed on air-dried calcium hydroxide. An upper red zone and a lower yellow zone (α-carotene) were formed. Elution of the red zone furnished a substance which on repeated crystallization from benzene-methanol gave glistening, reddish brown prisms of β-carotene, melting at 181–182°, with absorption maxima in carbon disulphide at 520, 484, 452 mμ, and in light petroleum at 484, 452, 426 mμ.

Filtrate 3. This, on evaporation, left a red, wax-like residue which was dissolved in light petroleum and rechromatographed on aluminium oxide. The upper red zone was eluted with light-petroleum methanol and the eluate concentrated to small bulk. On standing, the pigment separated in reddish needles which were still admixed with a colourless impurity. To remove the latter the pigment was extracted twice with boiling methanol and crystallized three times from benzene-methanol (2:1), when γ-carotene was obtained in dark red prisms, melting at 176–177°, with absorption maxima in carbon disulphide at 534, 496, 463 mμ and in chloroform at 508, 474, 446 mμ.

Methanol phase. This was diluted with water and extracted with light petroleum, all the pigment passing into the petroleum layer. This was washed free of methanol, dried with Na_2SO_4, and chromatographed on aluminium oxide. A faint reddish zone was formed which, on elution, gave a minute quantity of pigment with the absorption characteristics of γ-carotene.

SUMMARY

1. The pigments of the sponge *Hymeniacidon Sanguineum* (Grant) have been examined and found to be entirely epiphasic.

2. The carotenoids isolated and identified were α-, β- and γ-carotenes and echinenone.

3. No evidence of the presence of the crustacean pigment, astacene, was obtained.

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The Pigments of the Yellow Iris (*Iris pseudacorus*)

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It is of interest from the standpoint of phytochemical processes to record variations in the nature of the pigments among flowers of the same genus. Thus the yellow petals of *Crocus sativus* and the stigmas of the violet *C. neapolitanus* contain the carotenoid crocetin whereas the petals of the blue *C. evernus* owe their colour to an anthocyanin. A number of blue and purple varieties of the *Iris* family have been shown by Robinson & Robinson (1934) to contain specific anthocyanins whilst the present authors have already referred (O'Connor & Drumm, 1941) to the carotenoids of *I. pseudacorus*, the detailed examination of which forms the subject of the present communication.

I. pseudacorus, more commonly known as the waterflag, is found in marshy ground and flowers in June–July. The only recorded observation in the chemical literature concerning the pigments of this flower is that of van Wisselingh (1917). Using the well-known microchemical crystallization method of Molisch (1896), he established the carotenoid nature of the colouring matter but did not attempt the isolation of any individual pigment.

In the present work, a light-petroleum extract of the dried petals, after saponification, was fractionated between light petroleum and aqueous methanol, the pigments being resolved into hydrocarbons and a mixture of xanthophylls. By chromatographic resolution of the former, β-carotene was obtained in crystalline form. The xanthophylls of the methanol phase were separated by repeated adsorption on calcium carbonate into two crystalline pigments, that occupying the top zone being identified as violaxanthin, first isolated by Kuhn & Winterstein (1931) from the petals of the yellow pansy. The other pigment, occupying the middle zone, was identified as lutein (Kuhn, Winterstein & Lederer, 1931). These pigments may be present as esters in the flower. Spectroscopic evidence of the presence of yet a third xanthophyll, probably zeaxanthin, was obtained but the amount available was not sufficient for complete characterization.

EXPERIMENTAL

Yellow iris petals (2 kg.), collected in the month of June, were dried at 50°, powdered and sieved, giving a golden yellow powder (500 g.). This was covered with 2 l. of light petroleum (Analar, b.p. 30–50°) and allowed to stand overnight in an atmosphere of nitrogen. The light-petroleum extract was filtered off and the above treatment repeated until the extract was only faintly coloured. The combined orange-yellow extracts (6 l.) were concentrated to 2 l. at 40° *in vacuo*. The concentrated extract was then saponified by adding a solution of sodium ethoxide in ethanol (120 ml. of 10%), then sufficient ethanol (100 ml.) to make the solution homogeneous, and setting aside at room temperature for 24 hr. Methanol (90%) was added until a separation into two layers occurred. The lower layer was drawn off, and washed three times with light petroleum (100 ml.), the final washing being almost colourless.

The united light-petroleum extracts (Extract A) were next washed, once with 90% methanol (100 ml.), and then with a further two portions of 50 ml. each, all these washings being added to the main methanol layer. This methanol phase was then covered with a thin layer of light petroleum (b.p. 60–80°) and water carefully added until a distinct cloudiness appeared. On vigorous shaking, a solid began to separate and after the extract had stood overnight an orange-red solid had collected at the interface. This was collected, dissolved in carbon disulphide, the orange-red solution washed with water to remove traces of methanol, dried over anhydrous sodium sulphate, and chromatographed in portions on calcium carbonate columns (15 × 5 cm.). Developed with carbon disulphide the completed chromatograms exhibited the following zones:

Zone	Colour	Absorption maxima (CS_2) (mμ)
Top	Yellow	502, 470, 442
Middle	Orange-yellow	509, 476, 444
Bottom	Orange	515, 482, 450

Top zone. The eluate from this was brought to dryness *in vacuo*, the residue again dissolved in carbon disulphide and further resolved by readsorption on calcium carbonate. The resulting top band was eluted with methanol, the eluate evaporated to small bulk, covered with light petroleum and water added until a cloudiness resulted. The mixture was left overnight in the ice-chest and the solid which separated at the interface was collected and recrystallized several times from carbon disulphide, from which it was obtained as reddish brown needles, m.p. 198–199°; absorption maxima in carbon disulphide, 501, 469, 441 mμ and in chloroform 482, 451, 424 mμ. When dissolved in ether it gave a very intense blue colour reaction with 25% hydrochloric acid. With cold glacial acetic acid the crystals gave a grass-green colour, with dichloroacetic acid a blue, and when a drop of 70% perchloric acid was added to their solution in glacial acetic acid a deep blue colour was obtained. This pigment is therefore violaxanthin.

Middle zone. This was eluted with carbon disulphide containing ethanol and the latter then removed by repeated

washing with water. The washed solution was dried over anhydrous sodium sulphate and readsorbed on calcium carbonate. The completed chromatogram exhibited three definite bands, the uppermost of which was faintly yellow (violaxanthin). The middle orange-yellow band, which contained the main portion of the colouring matter, was eluted, and the eluate crystallized first from methanol and subsequently from carbon disulphide, the pigment being obtained as orange-red crystals, m.p. 194°; absorption maxima in carbon disulphide 511, 478, 446 mμ, in chloroform 487, 456, 427 mμ. The solution in concentrated acetic acid developed a yellow colour and in dichloroacetic acid the pigment dissolved to a greenish solution. This pigment is therefore lutein. The third pigmented zone at the base of the column was spectroscopically identical with the pigment contained in the bottom zone of the original chromatogram, to which it was therefore added.

Bottom zone. The eluate from this, which contained very little colouring matter, was readsorbed on calcium carbonate with the result that a separation from a further quantity of lutein was effected, but the quantity of pigment obtained was not sufficient for the isolation of any colouring matter; absorption maxima in carbon disulphide 516, 483, 451 mμ. It was probably zeaxanthin.

Extract A. The light-petroleum extract was washed free from methanol, dried over anhydrous sodium sulphate and adsorbed on aluminium oxide (Brockmann), the chromatogram being developed with light petroleum (b.p. 40–60°). The deep orange-red zone at the top of the column was eluted with benzene-ethanol and the solution concentrated to small bulk. After standing in the ice-chest for 24 hr. the precipitated pigment was repeatedly extracted with boiling ethanol to remove colourless impurities. Recrystallization of the residue from benzene gave reddish metallic-glancing prisms, m.p. 184°; absorption maxima in CS_2, 520, 484, 452 mμ and in petroleum ether, 485, 452, 424 mμ. This pigment is therefore β-carotene.

Ether extract. The deep red solution, obtained by extraction with diethyl ether of the methanolic mother liquors remaining after precipitation of the pigments by water, was washed free of alkali, dried over anhydrous sodium sulphate and the solvent removed *in vacuo*. The residual dark red oil product was dissolved in benzene and adsorbed on aluminium oxide, the chromatogram being developed with a 1:1 mixture of benzene and chloroform and finally with pure chloroform. The chromatogram contained two orange-red bands on top and a reddish diffuse band underneath. This latter band had no distinct absorption maxima and was not further examined. The top band, on elution, gave a dark red waxy solid; it showed absorption maxima in CS_2 at 497, 466, 437 mμ, in close agreement with the spectroscopic data for the unidentified lipochrome pigment isolated from *Viola tricolor* by Kuhn & Winterstein (1931).

The middle band of the above chromatogram had absorption maxima in CS_2 at 502, 470, 440 mμ. The pigment from this was dissolved in carbon disulphide and readsorbed on calcium carbonate. It gave two yellow bands very near the top of the column, the upper of which had absorption maxima in CS_2 at 497, 466, 437 mμ (identical with those of the waxy material above). The second and main band had absorption maxima at 501, 469, 440 mμ. The eluate from this gave the characteristic violaxanthin reaction with 25% hydrochloric acid.

SUMMARY

1. *Iris pseudacorus* has been examined and found to contain β-carotene and the xanthophylls violaxanthin and lutein.

2. A third xanthophyll, present in minute quantities, was spectroscopically identical with zeaxanthin.

3. In addition an uncrystallizable waxy carotenoid was present, similar in properties to that isolated by Kuhn & Winterstein (1931) from *Viola tricolor*.

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Addendum to paper by C. R. Harington & Rosalind V. Pitt Rivers (see p. 164)

The following information about the hydrates of *l*(+)-di-iodotyrosine is worth recording. Di-iodotyrosine crystallizes from water, either by separation from the hot solution or by neutralization of a solution in aqueous acid as the dihydrate which forms glistening plates, which retain their water of crystallization on air-drying. (Found: I, 53·9%; calc. for $C_9H_9O_3NI_2, 2H_2O$: I, 54·1%.)

On drying the above dihydrate over P_2O_5 *in vacuo* the water of crystallization is lost. (Found: I, 58·0%; calc. for $C_9H_9O_3NI_2$: I, 58·6%.)

Separation of di-iodotyrosine by evaporation of an ammoniacal solution at a bath temperature $> 40°$ yields long hair-like needles of the monohydrate. (Found: I, 56·1%; calc. for $C_9H_9O_3NI_2, H_2O$: I, 56·3%.)

When di-iodotyrosine separates slowly from 60% acetic acid it forms large prisms, containing one molecule of acetic acid, which are stable to desiccation *in vacuo* over P_2O_5. (Found: I, 51·5%; calc. for $C_9H_9O_3NI_2, C_2H_4O_2$: I, 51·5%.) When the separation is rapid the form obtained is the monohydrate described above.